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Synthesis of phosphonate analogues of ribose 1,5-bisphosphate and their effect on phosphofructokinase and fructose bisphosphatase

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Synthesis of Phosphonate Analogues of Ribose 1,5-Bisphosphate and Their Effect on Phosphofructokinase and Fructose Bisphosphatase

by

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ABSTRACT

Synthesis of Phosphonate Analogues of Ribose 1,5-Bisphosphate and Their Effect on Phosphofructokinase and Fructose Bisphosphatase

by

Gregory Linn

Adviser: Dr. Horst Schulz

Phosphonate analogues of α- and β-ribose 1,5-bisphosphate, in which the anomeric oxygen has been replaced by carbon, have been prepared in a multistep synthesis. The specific anomeric configuration of these purified compounds was determined using proton nuclear magnetic resonance and by analyzing fragments by mass spectrometry.

These analogues and α-ribose 1,5-bisphosphate were tested as effectors of rabbit muscle 6-phosphofructo-1-kinase and rat liver fructose 1,6-bisphosphatase. α-Ribose 1,5-bisphosphate and the α- and β-anomer bisphosphonate analogues activated 6-phosphofructo-1-kinase, with α-ribose1,5-bisphosphate activating the enzyme to its V_{max} and the α- and β-anomer analogues activating the enzyme to 80% and 30% of its V_{max}, res-

pectively. These results suggest that the analogues bind simultaneously to the allosteric and substrate sites. α–Ribose 1,5-bisphosphate and the α-anomer analogue inhibited fructose 1,6-bisphosphate non-competitively and did not potentiate adenosine monophosphate inhibition of this enzyme. The β-anomer analogue did not inhibit the enzyme even at a high concentration (> 1 mM). These results are consistent with inhibition occurring at a site distinct from the substrate or adenosine monophosphate allosteric site. However, in light of recent findings (Liang et al., 1992; see bibliography), this inhibition can be rationalized by binding of the former two compounds to either the adenosine monophosphate binding site or the substrate site.

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This thesis is dedicated to the memory of Dr. Donald Sloan. May this work be viewed as a continuation of Dr. Sloan's desire, as he said so many times, to "seek the truth".

This thesis is also dedicated to the memory of my father, Harry Linn. In this work I see my redemption in his eyes, and may he see his redemption in mine.

To Dr. Horst Schulz and Dr. Charlotte Russell I must give first thanks. Without their understanding of my desire to prove myself, and their letting me stay at City College to finish my synthesis, I could not have completed this degree.

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The stone rejected by the builders was used as the headstone.

Psalms 102:22

TABLE OF CONTENTS

APPROVAL PAGE....ii
ABSTRACT....iii
ACKNOWLEDGMENTS....v
PREFACE....vi
LIST OF TABLES....ix
LIST OF FIGURES....x
ABBREVIATIONS....xii
INTRODUCTION
Ribose 1,5-Bisphosphate....1
Hepatic Substrate Cycling....4
6-Phosphofructo-1-kinase and Fructose 1,6-Bisphosphatase....7
Sugar Bisphophate Effectors of Glycolysis and Gluconeogenesis....13
Hyperglycemia; Alleviation of Symptoms with Sugar Bisphosphates....17
RATIONALE....19

EXPERIMENTAL PROCEDURES....20
Materials and Methods....20
General Conditions....20
Assays for 6PF1K and Fru-1,6-P_2ase....21
Chemical Synthesis of Ribose Bisphosphate Analogues....22
1-Hydroxy-2*O*, 3*O*-isopropylidene-D-ribose-5-Pivaloate (**3a,3b**)....22
1-(Dimethoxyphosphinyl)-2*O*, 3*O*-isopropylidene-D-ribose-5-pivaloate (**4a,4b**)....23
α-or β-1-(Dimethoxyphosphinyl)-2*O*, 3*O*-isopropylidene-D-ribose (**5a,5b**)....24
α-or β-1-(Dimethoxyphosphinyl)-D-ribose-5-phosphate Dimethyl Ester (**6a** or **6b**)....25
α-or β-1-(Dihydroxyphosphinyl)-D-ribose 5-phosphate (**7a** or **7b**)....28
Discussion of Synthesis....30
Assignment of Configurations to Analogues **7a** and **7b**....30

Results of Biological Studies

Activation of Rabbit Muscle 6PF1K by Rib-1,5-P_2 and both α(**7a**)- and β(**7b**)-PRibCP....33

Inhibition of Rat Liver Fru-1,6-P_2ase by Rib-1,5-P_2 and PRibCP Analogues....34

Discussion of Biological Results....34

CONCLUSIONS....41

REFERENCES....72

LIST OF TABLES

Table 1. Summary of Kinetic Results for 6PF1K.....44
Table 2. Summary of Kinetic Results for Fru-1,6-P_2ase.....45

LIST of FIGURES

Figure

1. Regulation of Substrate Cycle Enzymes.....46

2. ^{1}H NMR spectrum of **5a**.....47

3. ^{13}C NMR spectrum of **5a**.....48

4. ^{1}H NMR spectrum of **5b**.....49

5. ^{13}C NMR spectrum of **5b**.....50

6. ^{1}H NMR spectrum of **6a**.....51

7. ^{13}C NMR spectrum of **6a**.....52

8. ^{31}P NMR spectrum of **6a**.....53

9. Mass spectrum (low resolution) of **6a**.....54

10. ^{1}H NMR spectrum of **6b**.....55

11. ^{13}C NMR spectrum of **6b**.....56

12. ^{31}P NMR spectrum of **6b**.....57

13. Mass spectrum (low resolution) of **6b**.....58

14. ^{1}H NMR spectrum of **7a**.....59

15. ^{13}C NMR spectrum of **7a**.....60

16. ^{31}P NMR spectrum of **7a**.....61

17. ^{1}H NMR spectrum of **7b**.....62

18. ^{13}C NMR spectrum of **7b**.....63

19. ^{31}P NMR spectrum of **7b**.....64

20. ^{1}H NMR spectrum of PRibPP.....65

21. Synthetic Scheme.....66

22. Possible Mechanism to Explain Fission Fragments.....67

23. Activation of 6PF1K by Rib-1,5-P_2 and α- and β-PRibCP.....68

24. Potentiation of AMP Activation of 6PF1K by Rib-1,5-P_2 and α- and β-PRibCP.....69

25. Inhibition of Fru-1,6-P_2ase by Rib-1,5-P_2 and α- and β-PRibCP.....70

26. Potentiation of AMP Inhibition by Rib-1,5-P_2 and α- and β-PRibCP.....71

ABBREVIATIONS

ara-1,5-P_2	Arabinose 1,5-Bisphosphate
CHA	Cyclohexylammonium
DMF	Dimethylformamide
NEM	*N*-Ethylmaleimide
Fru-6-P	Fructose 6-Phosphate
Fru-1,6-P_2	Fructose 1,6-Bisphosphate
Fru-1,6-P_2ase	Fructose 1,6-Bisphosphatase
Fru-2,6-P_2	Fructose 2,6-Bisphosphate
Glc-1,6-P_2	Glucose 1,6-Bisphosphate
Glt-1,3-P_2	Glycerate 1,3-Bisphosphate
HRMS	High Resolution Mass Spectrum
IPA	Isopropyl alcohol
ParaCP	Phosphoarabinosyl methylenephosphonate
6PF1K	6-Phosphofructo-1-kinase
PRibCP	Phosphoribosylmethylenephosphonate

Py	Pyridine
PRibPP	Phosphoribosylpyrophosphate
PTSA	*p* -Toluenesulfonic acid
Rib-1-P	Ribose-1-phosphate
Rib-1,5-P_2	Ribose 1,5-bisphosphate
TMSBr	Trimethylsilyl bromide
TLC	Thin layer chromatography
TMMBP	Tetramethylenemethylenebisphosphonate

INTRODUCTION

Ribose-1,5-Bisphosphate

Rib-1,5-P_2 was shown by Klenow (1) in 1953 to be synthesized from ribose 5-phosphate by the phosphoglucomutase reaction in the presence of glucose-1,6-bisphosphate. In 1970 Vanderheiden (2) showed it to be present in red blood cells. By 1974 Rose and Warm (3) demonstrated that Rib-1,5-P_2 activates 6PF1K. Guha and Rose (4) in 1986 suggested that the more likely origin of Rib-1,5-P_2 was the same synthase responsible for the synthesis of Glc-1,6-P_2. They showed that Rib-1,5-P_2 was synthesized in an extract of the cytosol of mouse brain in a reaction that used Rib-1-P as the acceptor for the acyl phosphoryl group of Glt-1,3-P_2. Glc-1,6-P_2 is synthesized in a similar reaction (5). However, not until1990 (6) was it shown that Rib-1,5-P_2 might actually have a role in vivo. In that study it was shown that glycolysis in a rat brain was activated several fold within a few seconds after the initiation of ischemia by decapitation. Analysis of metabolites in these

brain samples showed that within 2-5 seconds, Fru-6-P decreased by 60% and that Fru-1,6-P_2 increased seven fold, consistent with the activation of 6PF1K. According to this report, none of the known activators of 6PF1K in vivo, including Fru-1,6-P_2, Fru-2,6-P_2, Glc-1,6-P_2, or AMP could account completely for this rapid activation of the enzyme. However, a new activator of 6PF1K formed within 2-5 seconds after the activation of glycolysis in the brain. This time course corresponded exactly with the rate of activation of 6PF1K. This new activator was identified as Rib-1,5-P_2.

To date Rib-1,5-P_2 has been demonstrated to exist only in red blood cells and brain cells. However, the synthase suggested to be responsible for its synthesis is found also in muscle and liver cells (4). Its lack of detection in these and other cells may result from the possibility that it exists at very low levels. This is quite likely as it has been demonstrated that Fru-2,6-P_2, a well known effector of glycolysis and gluconeogenesis, exist at very low levels in skeletal muscle (7). Additionally, as shown earlier (6), it may

be that its synthesis is dependent on particular cellular conditions after which it quickly is degraded. Lastly, due to its lability, Rib-1,5-P_2 may become hydrolyzed during isolation from cell preparations.

In order to better ascertain whether Rib-1,5-P_2 does play a role in vivo, stable isosteric methylene phosphonate analogues of Rib-1,5-P_2 were synthesized. These analogues, both the α- and β-anomers, are considerably more resistant to hydrolysis than their bisphosphate counterparts. This is so because the phosphate ester of the latter compound has been replaced with a methylene phosphonate. This renders the resulting analogue resistant to not only general acid or base catalyzed hydrolysis but also to hydrolysis by intracellular phosphatases or esterases.

The following discussion is intended to focus, in particular, on the manner in which the known effectors of glucose metabolism act in vivo. The specific locations of the effects and the many factors which influence their activity provide important guideposts for the future study of the effects of these Rib-1,5-P_2 analogues on glucose metabolism.

Hepatic Substrate Cycling

The pathways of glycolysis and gluconeogenesis are the yin and yang of glucose metabolism, with the former pathway responsible for the catabolism of glucose and the latter pathway catalyzing the regeneration of glucose.

As one looks at the major enzymes in each of these pathways, it is also clear that there are oppositely functioning enzymes and that these can be considered as engaged in substrate cycling (Fig. 1). Glucose/glucose-6-phosphate is the first major substrate cycle and involves the enzymes glucokinase and glucose 6-phosphatase. Fru-6-P /Fru 1,6-P_2 is the second major substrate cycle and utilizes the enzymes 6PF1K and Fru-1,6-P_2ase. The third and last major substrate cycle in the gluconeogenic/glycolytic pathway is the phosphoenolpyruvate/pyruvate cycle in which pyruvate kinase is opposed by a multi-step conversion of pyruvate into phosphoenolpyruvate catalyzed by phosphoenol pyruvate carboxykinase and pyruvate carboxylase (8).

While glycolysis is operative in all organs of animals, gluconeogenesis functions largely in the liver. The kidney also has an equally high level of gluconeogenic activity. However, because of the kidney's small size, its contribution to overall blood glucose is rarely more than 10% of total body glucose synthesis (9). When the glucose level is higher than normal, the liver takes up the excess and stores it as glycogen or converts it to lipid by the process of glycolysis and lipogenesis. On the other hand, when blood glucose levels are less than normal, the liver produces glucose by the processes of glycogenolysis and gluconeogenesis. As discussed above, glycolysis and gluconeogensis share many of the same reversible or opposing enzymatic reactions. Yet the flux of intermediates through each of these two pathways is controlled by irreversible enzymes unique to either glycolysis or gluconeogensis. The net result of the operation of enzymes involved in these opposing reactions or substrate cycles would be the hydrolysis of ATP and the unnecessary expenditure of energy, as shown by

the equations below:

$$\text{Glucose} + \text{ATP} \longrightarrow \text{Glucose-6-P} + \text{ADP}$$
$$\text{Glucose-6-P} + H_2O \longrightarrow \text{Glucose} + P_i\ .$$

Similar equations can be written for the other two substrate cycles. One explanation for the existance of substrate cycles was by Newsholme and Gevers (10), who proposed that they were not energetically wasteful and that in fact the hydrolysis of ATP provided a mechanism for the amplification of allosteric control of flux through the pathways. One question about these substrate cycles is to what degree they function in vivo. Hue and Hers (11) using tritium labeled glucose gave a recycling rate at the glucokinase/glucose-6-phosphosphatase step of about 1µmol/g wet liver/min. Noting that this rate is probably much lower under gluconeogenic conditions (i.e. starvation), Clark et al. (12), also using tritium-labeled glucose, estimated the rate of recycling at the 6PF1K/Fru-1,6-P_2ase at about 0.20 µmol/g wet liver/min, using the hypoglycemic agent phenethylbiguanide. This rate under "physiological" conditions was only about 0.06 µmol/g wet liver/min. Finally, the rate of recycling at the pyruvate kinase/

phosphoenolpyruvate carboxykinase was estimated by Friedmann et al. (13) to be no more than 0.35 μmol/g wet liver/min.

The discovery of Fru-1,6-P_2ase activity in skeletal muscle (14,15) was unexpected, since its function was thought to be confined to gluconeogenesis. Newsholme et al. (16) after studying the flight muscle of the bumble bee, suggested that in some organisms Fru-1,6-P_2ase may act together with 6PF1K to catalyze a cyclic synthesis and hydrolysis of Fru-1,6-P_2, and thereby act as an ATPase for the production of heat. This hypothesis was confirmed by Bloxham et al. (17) and Clark et al. (18). They showed that there was extensive substrate cycling in non-flying bees, whereas during flight this cycling was completely absent, presumably because sufficient heat was generated as a byproduct of glycolysis.

6-Phosphofructo-1-kinase and Fructose-1,6-bisphosphatase

6PF1K (EC 2.7.1.11) catalyzes the phosphorylation of Fru-6-P to form

Fru-1,6-P_2;

$$\text{Fru-6-P} + \text{Mg·ATP} \rightleftharpoons \text{Fru-1,6-}P_2 + \text{Mg·ADP}.$$

This reaction is regarded as the first unique step of the glycolytic pathway, and therefore it is not surprising that the enzyme is subject to strong metabolic regulation. The reaction was first discovered in red cells by Dische (19) and in muscle by Ostern et al. (20). In general, procaryote phosphofructokinases are controlled by a small number of effectors, whereas phosphofructokinases from higher organisms are regulated by a multitude of metabolites. Furthermore, the latter enzymes undergo association-dissociation reactions and exist in various polymeric forms. Muscle 6PF1K can phosphorylate a number of sugar phosphates, including D-tagatose-6-P, D-fructose-1-P, D-glucose-1-P, D-seduheptulose-1-P and D-fructose-6-sulfate (21). Koerner et al. (22) and Bar-Tanna and Cleland (23) showed that 2,5-anhydromannitol-6-P, an analogue of β-D-Fru-6-P, served as a substrate but that 2,5-anhydroglucitol-6-P, an analogue of α-D-Fru 6-P, was an inhibitor. This confirmed earlier observations of Wurster and Hess (24) who used the stopped-flow kinetic tech-

nique to conclude that the β-anomer was the only active form of Fru 6-P used by the enzyme. It is interesting to point out that Fru-1,6-P_2-ase has a specificity for the α-anomer of Fru-1,6-P_2 (25).

Like all kinase reactions, 6PF1K requires Mg^{2+} for its activity and the ATP-divalent cation complex is the active substrate. The Mn^{2+} and Co^{2+} ions are also active for this enzyme. Additionally, 6PF1K is also activated by NH_4^+ and K^+ (16).

6PF1K is an allosteric enzyme, and as such its activity is modulated by a variety of effectors. In general all phosphofructokinases from higher organisms show the following properties of regulation:

1. Inhibition by a high concentration of ATP.
2. Inhibition by citrate at an inhibitory concentration of ATP.
3. Relief of the inhibition caused by ATP by P_i, AMP, cAMP, ADP, Fru-6-P, Fru-1,6-P_2, Fru-2,6-P_2 and Rib-1,5-P_2.
4. Cooperativity of Fru 6-P binding. Negative effectors decrease affinity for Fru 6-P and positive effectors increase the affinity.

The inhibition of rabbit muscle 6PF1K by ATP was discovered by Lardy and Parks (26). The biological significance of this inhibition becomes clear in light of the fact that glycolysis serves to generate ATP in anaerobic cells and also provides oxidizable metabolites for mitochondria and aerobic cells. Further, the fact that the degradative products of ATP (i.e., ADP, AMP, and P_i) relieve ATP inhibition supports the importance of regulation of phosphofructokinase activity by the energy status of a cell.

The pH of the medium plays an important role in the ATP inhibition of 6PF1K. It was initially shown with the rabbit muscle enzyme that ATP is a strong inhibitor at pH below 7.1, a weaker inhibitor at pH 7.5-8.5, and is not inhibitory at all at pH 9 (27).

Citrate is another important inhibitor of 6PF1K activity. Interestingly, citrate only inhibits in the presence of an inhibitory concentration of ATP (28). Of the positive effectors of 6PF1K, the most potent is Fru-2,6-P_2. It is about 10 times more potent than the next best activator, Rib-1,5-P_2 (29). Glc-1,6-P_2 and Fru-1,6-P_2 are also important

activators. Additionally, Ishikawa et al. (65) have demonstrated that several acyclic sugar bisphosphates also activate 6PF1K. Muscle 6PF1K displays sigmoidal kinetics. Qualitatively, positive effectors shift the curve to the left, indicating an increase in affinity for Fru 6-P, whereas negative effectors more the curve to the right, indicating a decrease in affinity for substrate. The aggregation state of 6PF1K may also influence its kinetic behavior. Reinhart and Lardy (30) found that rat liver enzyme gave nonlinear rates of activity when it was diluted, whereas linear rates were observed when high concentrations of enzyme were used.

Based on several techniques including sedimentation equilibrium studies and SDS polyacrylamide gel electrophoresis the active form of 6PF1K is a tetramer having a molecular weight of 320,000-360,000 (31).

Fructose-1,6-bisphosphatase (EC 3.1.3.11) was first described by Gomori (32). The enzyme preparations described by Gomori were characterized by an alkaline pH optimum, and showed little or no activity in the neutral pH range. Subsequently, evidence began to accumulate indicating

that this pH optimum was not an intrinsic property of the enzyme but more likely an artifact of the procedure used to purify it. Experiments by Pogell and McGilvery (33) and others all pointed to modification by endogenous proteolytic activity as the cause of the shift in pH optimum during isolation of the enzyme from rabbit liver. Neutral Fru-1,6-P_2ase was then isolated by steps involving heating at neutral pH and chromatography on phosphocellulose, using the substrate elution procedure introduced by Pogell (34). Neutral Fur-1,6-P_2ases purified from rabbit liver and kidney were shown to be homogeneous proteins with a molecular weight of approximately 140,000 (35). Each was composed of four subunits having molecular weight weights of 35,000-36,000, as determined by SDS gel electrophoresis or sedimentation equilibrium measurements.

Fru 1,6-P_2ase appears to activated by a number of metal ion chelators. Van Tol et al. (36) showed that both a free divalent cation and metal chelate of this cation are required for the activity of Fru-1,6-P_2ase at

neutral pH. EDTA, phosphocreatine, histidine, and citrate are the major chelators which activate the purified enzyme. The latter two chelators are also physiological metabolites that can regulate the activity of Fru-1,6-P_2ase through the formation of metal chelates.

Fru-1,6-P_2ase is subject to allosteric inhibition by AMP (37) and substrate inhibition by Fru 1,6-P_2 (38). It is competitively inhibited by Fru-2,6-P_2 (39) and shows noncompetitive inhibition by Rib-1,5-P_2 (29). It is also inhibited by Zn^{2+} and Ca^{2+} (40).

Sugar Bisphosphate Effectors of Glycolysis and Gluconeogenesis

Fru-2,6-P_2 is the most potent sugar bisphosphate regulator of the 6PF-1K /Fru-1,6-P_2ase substrate cycle. It was discovered during studies on the hormonal regulation of hepatic 6PF1K activity. Addition of glucagon to hepatocytes resulted in the inhibition of 6PF1K activity, and since this addition correlated with an increase in phosphorylation of the kinase, it was initially thought that this was the cause of the decrease in kinase activity.

However, there was no correlation between increases in glucagon concentrations and decreases in enzyme activity. Furthermore, this hormonal effect was abolished upon partial purification of the enzyme, suggesting that enzyme activity was due to changing levels of some effector (41). Lastly, Reinhart and Lardy (30) examined the kinetic behavior of purified rat liver 6PF1K using physiological amounts of its known effectors (AMP and ATP) and its substrate, Fru-6-P. The resulting activity was not high enough to account for the glycolytic flux observed in vivo. The discovery of Fru-2,6-P_2 (43-45) completely accounted for this discrepancy. The regulation of Fru-2,6-P_2 synthesis has been reviewed extensively elsewhere (46-48), and hence will only be discussed here in very broad terms.

Glc-1,6-P_2 is present in liver and additionally is a potent regulator of carbohydrate metabolism in muscle and other extrahepatic tissues some of which are lung, brain, kidney, and testes (49). Levels of Glc-1,6-P_2, like those of Fru-2,6-P_2, are controlled hormonally or by cAMP. Epinephrine acts to increase levels of Glc-1,6-P_2 mediated by increased

levels of cAMP (50). This hormone, on the other hand, causes an increase of Fru-2,6-P_2 mediated by Ca^{2+}-induced activation of phosphorylase kinase (51).

Outside the liver Fru-2,6-P_2 plays little or no role in the regulation of glycolysis. In muscle cells incubated with epinephrine or insulin or in starved and then refed cells, Glc-1,6-P_2 levels increased whereas Fru-2,6-P_2 remained constant (52). Similarly, in muscle cells made diabetic or in starved cells Glc-1,6-P_2 levels decreased with no change in Fru-2,6-P_2 concentration (53). Glc-1,6-P_2 may be considered a "global" effector of glycolysis since it regulates at least six enzymes in this pathway, including hexokinase, 6PF1K, Fru-1,6-P_2ase, and pyruvate kinase (52), whereas only 6PF1K and Fru-1,6-P_2ase are regulated directly by Fru-2,6-P_2 in either hepatocytes or extrahepatic cells. Unfortunately, there seems to be no indication in the literature as to whether Glc-1,6-P_2 has a similar function in hepatocytes.

Of all the factors which influence the levels of Fru-2,6-P_2 and Glc-1,6-P_2,

one seems to affect both in the same direction. Under anoxic conditions in all types of cells, the levels of both of these effectors are greatly decreased (52,53). This observation is especially intriguing since Ogushi et al. (6) observed a transient but large increase in Rib-1,5-P_2 levels in rat brain made ischemic. Moreover, Guha and Rose (4) noted that Fru-2,6-P_2 levels decrease in hepatocytes made anoxic and that under this condition, Rib-1-P would be generated and possibly lead to an increase in the Rib-1,5-P_2 level.

Fru-1,6-P_2 has been shown to be an important effector of the third hepatic substrate cycle, pyruvate/phosphoenolpyruvate. This cycle is the rate limiting portion of gluconeogenesis and there is much evidence that pyruvate kinase is a major determinant of the rate of this pathway (8). Here, Fru-1,6-P_2 acts as a positive allosteric effector of pyruvate kinase. However, Fru-2,6-P_2 may exert indirect control. Decreased levels of Fru-2,6-P_2 cause a decrease in activity of 6PF1K and hence a decrease in the level of Fru-1,6-P_2. This, of course, will cause a decrease in pyruvate kinase activity and hence increase glucose synthesis and depress glyco-

lysis. There are also other factors affecting pyruvate kinase activity. Glucagon, as mentioned earlier, depresses Fru-2,6-P_2 levels and at the same time causes cAMP-mediated phosphorylation of pyruvate kinase which inhibits the enzyme.

Hyperglycemia; Alleviation of Symptoms by Sugar Bisphosphates

Gluconeogenesis is a process by which glucose is produced from various noncarbohydrate precursors such as alanine, glycerol or lactate and whose function is to prevent the level of blood glucose from falling below normal. Blood glucose is essential for the functioning of tissues that depend exclusively on it for energy supply. The major such organ is brain. Also, red blood cells depend on glucose.

Hepatic gluconeogenesis is hormonally regulated. Glucagon seems to be the most important hormone to stimulate this process. Only insulin, however, seems to play a major inhibitory role. The primary function of glucagon is to prevent hypoglycemia. In fact hypoglycemia is a potent stimulus for glucagon secretion.

The effects of glucagon are counteracted by insulin and, apparently,

it is the glucagon-to-insulin ratio rather then the absolute level of each hormone that determines the metabolic responses of the target organs (54).

A major physiological function of insulin is to prevent hyperglycemia. This is attained by inhibiting glucose release from the liver and by stimulating its peripheral utilization, that is, away from the central nervous system (55).

One result of the elucidation of how sugar bisphosphates affect the reglation of glucose metabolism has been the attempt to design inhibitors of gluconeogenesis. To this end, Hanson et al. (56) found that 2,5-anhydro-D-mannitol, an analogue of β-fructose, decreases blood glucose by 17 to 58% in fasting mice. This effect was attributed to the conversion of this analogue into either the mono-or bisphosphate. The bisphosphate analogue, in particular, was shown to activate pyruvate kinase and inhibit Fru-1,6-P_2ase. Several different analogues of Fru-2,6-P_2 were made in an attempt to inhibit Fru-1,6-P_2ase in hepatocytes but were unsuccessful (57,58).

RATIONALE

The reason for making phosphonate analogues of Rib-1,5-P_2 was to determine if they would mimic the kinetic behavior of Rib-1,5-P_2. If so, these analogues could serve as stable probes to investigate the in vivo role of Rib-1,5-P_2. Since 6PF1K and Fru-1,6-P_2-ase were known to be activated or inhibited by several different sugar bisphosphates, including Rib-1,5-P_2, it was reasonable to assume that the structurally related methylene phosphonate analogues would work as effectors of these enzymes as well.

The specific experiments used were taken from Pilkis et al. (70), and are described in detail in the experimental section.

EXPERIMENTAL PROCEDURES

MATERIALS AND METHODS

CHA Rib-1,5-P_2 was a gift from Dr. H. G. Khorana (MIT). ATP, AMP, Fru-6-P, Fru-1,6-P, Fru 2,6-P_2 were obtained from Sigma Chemical Company. Rabbit muscle 6PF1K, aldolase, glycerol phosphate dehydrogenase, triose phosphate isomerase, phosphoglucoisomerase and glucose 6-phosphate dehydrogenase were obtained from Boehringer Mannheim Company.

Rat liver Fru-1,6-P_2ase was purified according to Pilkis et al. (59). TMMBP was purchased from Lancaster Synthesis (Windham, NH). Dimethoxychlorophosphate was made (see ref. 62) by reacting dimethyl phosphite with sulfuryl chloride and characterized by IR spectroscopy. All other materials were obtained from Aldrich Chemical Company.

GENERAL CONDITIONS

Where appropriate, reactions were performed under anhydrous conditions. Dimethylformamide was dried over phosphorous pentoxide and distilled. Ether was dried over sodium metal and used directly. Methanol was dried by adding sodium metal and then distilling. Methylene chloride was purified by washing with a 5 % aqueous sodium carbonate solution, followed by water,

dried with calcium chloride and distilled. Pyridine was dried by the addition of calcium hydride and used directly. Dry THF was prepared by adding sodium metal, refluxed in the presence of benzophenone, and distilled.

Column chromatography was performed using 230-400 mesh silica gel from E. Merck. TLC was done on 0.25-mm thick silica gel GF Analtech Uni-plates (Newark, DE). Spots were visualized in an iodine chamber or by spraying with sulfuric acid followed by charring on a hot plate.

Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on an IBM NR300 spectrometer. ^{13} C and ^{31}P Nuclear magnetic resonance (^{13}C NMR, ^{31}P NMR) spectra were recorded on a Nicolet NTC360 spectrometer operating at 90 MHz or 145.2 MHz (H_3PO_4 external refererence), respectively.

Mass spectra were performed on a Finnigan DSQ 70 or a VG7070E spectrometer. IR spectra were recorded on a Perkin-Elmer 247 grating spectrophotometer. Optical rotations were measured on a Jasco DIP-181 polarimeter. Elemental analyses were obtained from Desert Analytics (Tucson, AZ).

Assays for 6PF1K and Fru-1,6-P_2ase

Activation of 6PF1K, studied by using a coupled enzyme assay, was monitored by the decrease in absorbance of NADH at 340 nm according to

Pilkis et al. (59). Inhibition of rat liver Fru-1,6-P_2ase, using a coupled enzyme assay, was monitored by the increase in absorbance of NADPH at 340 nm according to the method of El-Maghrabi et al. (60).

CHEMICAL SYNTHESIS OF RIBOSE BISPHOSPHATE ANALOGUES

Compounds **2a** and **2b** were made according to ref. 61 without any modification. MS for $C_8H_{14}O_5$ (170), m/z (c.i.) 171 (M + 1).

1-Hydroxy-2*O*, 3*O*-isopropylidene-D-ribose 5-Pivaloate (3a,3b).

Twelve grams (63 mmol) of mixture **2a,2b** (prepared from ref. 61) were dissolved in dry pyridine (150 ml). To this solution was added trimethylacetyl chloride (7.61 g, 63 mmol). The solution was stirred overnight at room temperature. Pyridine was removed under reduced pressure. The residue was dissolved in 100 ml of chloroform and extracted with 50 ml of a 10% sodium bicarbonate solution. The organic phase was recovered and dried over $MgSO_4$. After filtration, the solution was concentrated to10 ml and applied to a silica gel column. The compounds were eluted with cyclohexane/EtOAc, (3:1) and the desired product was obtained as a gum (R_f 0.65). Yield:14.6 g (84%). MS for $C_{13}H_{22}O_6$ (274.1), m/z (c.i.) 257 (MH^+-H_2O).

1-(Dimethoxyphosphinyl)-2*O*, 3*O*-isopropylidene-D-ribose 5-Pivaloate (4a, 4b). To a hexane-washed suspension of sodium hydride (1.1 g, 44 mmol) was added 150 ml of dry THF. While stirring, a solution of TMMBP (10.0 g, 44 mmol) in 50 ml of dry THF was added dropwise over a 20-min period at room temperature. The solution was stirred until it reached room temperature, at which time a solution of **3a,3b** (8.0 g, 136.5 mmol) in 50 ml of THF was added all at once. The reaction was allowed to proceed for 2.5 hr. The reaction was quenched with 70 ml of a saturated NH_4OAc solution. The reaction mixture was poured into a separatory funnel and the organic phase was removed. The aqueous phase was extracted with EtOAc (2x30 ml) and the organic layers were combined, dried ($MgSO_4$), and the solvent was removed under reduced pressure. Chromatography on silica gel (Et_2O/CH_2Cl_2, 5:2) yielded starting sugar **3a,3b** (1.5 g, R_f 0.7), product **4a,4b** (8.7 g, R_f 0.6), and unreacted TMMBP. Yield: 8.7 g (84%) based on recovered starting material. MS for $C_{16}H_{29}O_8$ P(380. 19), m/z (c.i.) 381 (M+1).

α-or β-1-(Dimethoxyphosphinyl)-2*O*, 3*O*-isopropylidene-D-ribose (5a,5b). To 100 ml of dry methanol was added 580 mg (25 mmol) of sodium. After the metal disappeared, a solution of the mixture **4a,4b** (8.6 g, 22.6 mmol) in 30 ml dry methanol was added all at once. The solution was stirred for 1.5 hr, after which time the TLC ($CHCl_3$/MeOH, 98:2) showed that no starting material remained. The reaction was quenched with 30 ml of saturated aqueous NH_4OAc solution. The methanol was removed under reduced pressure, and the residue in the flask was dissolved in chloroform. The flask was washed with water, and the aqueous and organic phases were combined. The organic phase was isolated using a separatory funnel and the aqueous phase was extracted with chloroform (2x30 ml). The organic phases were combined, dried ($MgSO_4$), and concentrated to10 ml. The solution was loaded onto a silica gel column and chromatographed in the above mentioned solvent system. The elution of both products was verified by ^1H NMR since tailing on TLC plates made visualization of the separated compounds difficult. Yield: 2.1 g (50%) of **5a** and 1.2 g (45%) of **5b**.

5a: **Fig. 2**: ^1H NMR ($CDCl_3$): δ 1.34 and1.49 (3H each, s, CH_3), 2.23 (2H, dd,

C(1)H_aH_b), 3.62 (2H, m, C(6)H_aH_b), 3.76 (6H, app. t, OCH_3), 4.14 (1H, t, C(5)H), 4.33 (1H, m, C(2)H), 4.69 (2H, m, C(3)H and C(4)H). **Fig. 3**: ^{13}C NMR ($CDCl_3$): 25.42 (C1, J_{CP} = 150.6 Hz), 81.75 (C2, J_{CCP} = 8.25 Hz), 75.77 (C3), 82.77 (C4), 84.58 (C5), 62.09 (C6), isopropylidene: 24.99 and 26.19 (CH_3), 112.38 (ketal C); 51.10 and 52.60 (OCH_3, J_{COP} = 6.75 Hz). IR (neat) cm^{-1} 3450, 3370, 1620, 1460, 1380, 1240, 1150, 830. MS for $C_{11}H_{21}O_7P$ (296.12), m/z (f.a.b.) 297 (M+1).

5b: Fig. 4: ^1H NMR ($CDCl_3$): δ 1.34 and 1.53 (3H each, s, CH_3), 2.24 (2H, m, C(1)H_aH_b), 3.63 (1H, dd, C(6)H), 3.82 (1H, dd, C(6)H), 3.76 (6H, app. t, OCH_3), 4.17 (1H, m, C(5)H), 4.25 (1H, dqt, C(2)H), 4.62 (1H, app. t, C(3)H), 4.80 (1H, dd C(4)H). **Fig. 5**: ^{13}C NMR ($CDCl_3$): 29.25 (C1, J_{CP}=142.4 Hz), 82.52 (C2, J_{CCP}= 5.85 Hz), 80.00 (C3), 83.10 (C4), 85.00 (C5), 67.10 (C6), isopropylidene: 25.35 and 27.50 (CH_3),114.82 (ketal C); 52.07 and 52.69 (OCH_3, J_{COP} = 6.31 Hz). IR (neat) cm^{-1} 3450, 3370, 1620,1460, 1380, 1240, 1150, 830. MS for $C_{11}H_{21}O_7P$ (296.12), m/z (f.a.b.) 297 (M+1).

α-or β-(Dimethoxyphosphinyl)-D-ribose-5-phosphate Dimethyl Ester (6a,6b). Preparation of **6a**: To **5a** (1.3 g, 4.4 mmol) was added

anhydrous pyridine (10 ml) and the solvent was removed under reduced pressure. After this procedure was repeated, dry ether (10 ml) was added, followed by dry pyridine (2.1 g, 26 mmol). While stirring, dimethyl chlorophosphate (1.9 g,13.2 mmol, bp 75-76°C/20mm Hg, prepared according to ref. 62) was added by glass micropipette all at once. The reaction proceeded overnight at room temperature. The precipitated pyridinium chloride was removed by filtration and washed with dry ether. The ether was removed under reduced pressure, and the residue was chromatographed on silica using EtOAc/IPA,10:1. Yield: 300 mg (17%).

Fig. 6: ^{1}H NMR ($CDCl_3$): δ 1.34 and 1.49 (3H each, s, CH_3), 2.24 (2H, m, C(1)H), 3.77 (6H, m, OCH_3), 4.07 (2H,m, $C(6)H_aH_b$), 4.23 (1H, m, C(5)H), 4.32 (1H, m, C(2)H), 4.7 (1H, dd, C(3)H), 4.78 (1H, app. t, C(4)H).

Fig. 7: ^{13}C NMR ($CDCl_3$): δ 25.63 (C1, J_{CP} = 140.85 Hz), 82.53 (C2, J_{CCP} = 7.73 Hz), 76.76 (C3), 81.78 (C4, J_{CCCOP} = 7.33 Hz), 82.69 (C5), 67.19 (C6, J_{COP} = 6.08 Hz), 26.39 and 25.18 (CH_3), 112.94 (ketal), 54.523 (OCH_3, J_{COP} = 5.54 Hz), 52.64 (OCH_3, J_{COP} = 5.85 Hz), 52.24 (OCH_3, J_{COP}= 3.75 Hz). **Fig. 8**: ^{31}P NMR($CDCl_3$): δ 31.487 (s, phosphinyl P), 1.854 (s,

phosphoryl P). IR (neat) cm^{-1} 3425, 3000, 2970, 2850, 1640, 1460, 1390,1380, 1260, 1240, 1220, 1180, 1160, 1030, 900, 860, 820. HR MS for $C_{13}H_{26}O_{10}P_2$ (404.1001), m/z (c.i.) 405.0983 (M+1). **Fig. 9** Low resolution MS (see discussion on page 31).

Preparation of **6b**: One gram (3.38 mmol) of **5b** was reacted as described above. Yield: 330 mg (24%). **Fig. 10**: ^1H NMR ($CDCl_3$): δ 1.34 and 1.53 (3H each, s, CH_3), 2.15 (2H, m, $C(1)H_aH_b$), 3.77 (6H, m, OCH_3), 3.77 (2H, m C(6)H) 4.15 (1H, m, C(5)H), 4.24 (1H, m, C(2)H), 4.52 (1H, dd, C(3)H), 4.65 (1H, dd, C(4)H). **Fig. 11:** ^{13}C NMR ($CDCl_3$): δ 30.01 (C1, J_{CP} = 140.55 Hz), 82.94 (C2, J_{CCP} = 7.28 Hz), 80.00 (C3), 81.76 (C4), 85.36 (C5, J_{CCOP} = 10.58 Hz), 67.39 (C6, J_{COP} = 5.70 Hz), 114.89 (ketal C), 27.45 and 25.63 (CH_3), 54.44 (OCH_3, J_{POC} = 5.25 Hz), 52.46 (OCH_3, app. t, J_{POC} = 7.28 Hz). **Fig. 12**: ^{31}P NMR ($CDCl_3$): δ 30. 221(s, phosphinyl P), 2.030 (s, phosphoryl P). IR (neat) cm^{-1} 3425, 3000, 2970, 2850, 1640,1460,1390, 1380,1260, 1240, 1220, 1180, 1160,1030, 900, 860, 820. HR MS for $C_{13}H_{26}O_{10}P_2$ (404.1001), m/z (c.i.) 405.1049 (M+1). **Fig. 13:** Low resolution MS (see discussion on page 31).

α-or β-1-(Dihydroxyphosphinyl)-D-ribose 5-Phosphate (7a, 7b). To **6a** (260 mg. 0.64 mmol) was added 10 ml of dry methylene chloride. While stirring neat TMSBr (492 mg, 3.2 mmol) was added. After 45 min the ^1H NMR spectrum showed the complete absence of methyl ester signals. The volatiles were removed under reduced pressure. The residue was dissolved in 10 ml of water and this solution was placed in a water bath at 60°C for one hour. At this time the ^1H NMR spectrum showed the complete absence of acetonide signals. Water was removed under reduced pressure. The residue (**7a**) was dissolved in methanol (25 ml) and the solution was neutralized with concentrated NH_4OH. The resulting precipitate was filtered and washed with methanol (10 ml). The precipitate was collected and excess methanol was removed under reduced pressure. The triammonium salt was dried overnight in a vacuum desiccator with P_2O_5. Yield: 230 mg (100%). $[\alpha]_D^{23}$ -6.18° (*c* 1.33, H_2O). **Fig. 14**: ^1H NMR (D_2O): δ 1.75 (2H, ddd, C(1)H_aH_b), 3.09 (1H, s, phosphonate) 3.63 (1H, m, C(4)H), 3.72 (2H, m, C(6)H), 3.95 (1H, m, C(3)H), 4.02 (1H, m, C(2)H), 4.03 (1H, m, C(5)H). **Fig. 15**: ^{13}C NMR (D_2O): δ 28.81 (C1, J_{CP}

=109.4 Hz), 72.53 (C2, J_{CCP} = 69.67 Hz), 77.63 (C3, J_{CCCP} = 67.7 Hz), 77.63 (C4), 80.44 (C5, J_{CCOP} = 24.9 Hz), 64.97 (C6, J_{COP} =10.5 Hz). **Fig. 16**: ^{31}P NMR (D_2O): δ 20.136 (s, phosphinyl P), 2.473 (s, phosphate ester). IR (KBr) cm^{-1} 3150,1642,1400, 1125, 1050, 980, 915, 800. Anal. Calcd for the triammonium salt, $C_6H_{23}O_{10}P_2N_3$ (359.09): C, 20.06; H, 6.46; P, 17.26. Found: C, 20.05; H, 6.45; P, 17.00.

The triammonium salt of 7**b** was prepared from **6b** (320 mg, 0.792 mmol) as described above. Yield: 280 mg (100%). $[\alpha]_D^{23}$ + 3.7° (*c* 2.75, H_20). **Fig. 17**:^1H NMR (D_2O): δ 1.60 (1H, m, C(1)H_a), 1.75 (1H, m, C(1) H_b), 3.09 (1H, s, phosphonate), 3.60 (2H, m, C(6)H), 3.68 (1H, app. t, C(4)H), 3.74 (1H, m, C(3)H), 3.80 (1H, m, C(2)H), 3.95 (1H, t, *J* = 4.80 Hz C(5)H). **Fig. 18**: ^{13}C NMR (D_2O): δ 37.53 (C1, J_{CP} = 126.22 Hz), 79.36 (C2, J_{CCP} = 5.56 Hz), 75.58 (C3), 83.15 (C4), 86.85 (C5, J_{CCOP} = 8.08 Hz), 68.51 (C6, J_{COP} = 4.40 Hz). **Fig. 19**: ^{31}P NMR (D_2O): δ 19.188 (s, phosphinyl P), 3.895 (s, phosphate ester). IR (KBr) cm^{-1} 310,1642, 1400, 1125, 1050, 980, 915, 800. Anal. Calcd for the triammonium salt $C_6H_{23}O_{10}P_2N_3 \cdot H_2O$

(377.153); C,19.09; H, 6.68; P, 16.42. Found: C,19.59; H, 6.91; P,16.09.

DISCUSSION of SYNTHESIS

ASSIGNMENT of CONFIGURATIONS to ANALOGUES **7a** and **7b.**

Fig. 21 outlines the synthetic route used to make **7a** and **7b.** Starting with suitably protected ribose (**3a,3b**) an Emmons-Horner reaction, using TMMBP, resulted in compounds **4a** and **4a** (63,64). These compounds could not be separated. However, after the removal of the trimethylacetyl group, which yielded **5a** and **5b**, the two anomers were separated by column chromatography. The separation was followed by ^1H NMR because sufficient resolution could not be achieved on TLC plates, due to tailing. Since each anomer has two ^1H acetonide signals, and the downfield acetonide signal of each anomer is different (Figs. 2 and 4), anomeric purity was established by isolating only those column fractions that contained a unique downfield acetonide signal. These two purified intermediates were then phosphorylated (yielding **6a** and **6b**) using dimethyl chlorophosphate, purified, and their configurations were determined.

Ohrui et al. (65) have established that for fully protected ribofuranosyl C-glycosides, all the ring protons chemical shifts of the α-anomers are downfield from those of the β-anomers. Moreover, the difference in the chemical shifts of the α-anomer acetonide methyl groups (Δ ppm) should be smaller than those of the β-anomer (65). One of the anomers that had these downfield ring proton chemical shifts was shown by X-ray crystallography to have the C-glycoside moiety below the plane of the ribose ring. Also, the anomer with upfield ring proton chemical shifts showed its C-glycoside moiety to be above the plane of the ribose ring. The proton NMR data (Figs. 6 and 10) are consistent with assigning to **6a** (Δ 0.15 ppm) the α configuration and to **6b** (Δ 0.18 ppm) the β configuration.

This assignment was confirmed by mass spectrometry. Fragments from **6b** (Fig. 13) were consistent with the fission of the phosphate ester at C(6), whereas no such fragments resulted from **6a** (Fig. 9). That this result is a function of structure and not bond strength was confirmed by collision activation analysis (63) which puts energy into the system. The excess energy, which is the result of raising the gas pressure of the system from 10^{-7} to 10^{-3} torr, did not affect the fragment pattern of either compound.

The fragment pattern of **6b** can be explained most readily by a rearrangement reaction in which a proton from C(1) migrates to the C(6) oxygen resulting in the fission of the phosphate diester. This cannot happen with the α-anomer (Fig. 22). Compounds **6a** and **6b** were deesterified using TMSBr and the acetonide group was removed simultaneously with acid, yielding **7a** and **7b** quantitatively. The dramatic upfield shift of C(1) of **5a** (Fig. 3) relative to that of **5b** (Fig. 5) and even more so the upfield shifts of C(1)-C(3) of **6a** (Fig. 7) versus those of **6b** Fig. 11) are indicative of a more stable conformation for the "**a**" compounds (ref. 66, page 4046).

The ^{31}P NMR spectra of **6a** (Fig. 8) and **6b** (Fig.11) were recorded using gated decoupling in order to assure the proper integration of the phosphorous signals. In contrast to the results of McClard and Witte (65), the ^{31}P NMR spectra of **7a** (Fig. 16) and **7b** (Fig. 19) do not show the formation of a phostone (around 40 ppm) after removal of the acetonides. The reason for this difference is unclear. The chemical shifts in the ^{13}C NMR spectra for **7a** (Fig. 15) and **7b** (Fig. 18) are consistent with the

previous series of carbon spectra. A less exact, but suggestive confirmation of the assignments of configurations, is to look at the ^1H splitting of **7a** (Fig. 14) versus that of **7b** (Fig. 17). The former has a splitting pattern quite similar to that of PRibPP (Fig. 20), whereas **7b** shows a radically different splitting pattern.

By replacing the phosphate of Rib-1,5-P_2 with a methylenephosphonate, the acidity of this moiety has been greatly reduced. Integration of proton NMR signals of the de-esterified triethylammonium salt of **5a** or **5b** (data not shown) shows only one triethylamine group per sugar molecule. This has also been seen by McClard and Witte for the analogous tetrabutylammonium compounds (65) and suggests a high pK_a for **7a** or **7b.**

RESULTS of BIOLOGICAL STUDIES

Activation of Rabbit Muscle 6PF1K by Rib-1,5-$P_2$and both α (7a)-and β (7b)-PRibCP. Rib-1,5-P_2 and α– or β–PRibCP both activated rabbit muscle 6PF1K. The results are shown in Fig. 23 and are summarized in Table 1. Rib-1,5-P_2 (K_A = 3.5 μM) was almost twice as effective as α–PRibCP (K_A= 6.5 μM) and at least fifteen times as effective as β–PRibCP (K_A> 52 μM). Fru-2,6-P_2 (K_A= 0.086 μM) was by far the most effective activator. Rib-1,5-P_2 and the PRibCP analogues

also potentiated AMP activation of 6PF1K (Fig. 24). The K_A for AMP was reduced to 5.0 μM from 10.0 μM in the presence of β-PRibCP and to 2.5 μM in the presence of either Rib-1,5-P_2 or α–PRibCP.

Inhibition of Rat Liver Fru-1,6-P_2 by Rib-1,5-P_2 and PRibCP Analogues. Contrary to earlier reports (66,71), Rib-1,5-P_2 did inhibit Fru 1,6-P_2ase. α-PRibCP also inhibited this enzyme. These results are summarized in Table 2. The maximum decrease in enzyme activity caused by both these compounds was 50%. Fig. 25 shows that this inhibition was independent of Fru-1,6-P_2 concentration and that they both have a virtually identical K_{IS} (87 μM). Furthermore, both Rib-1,5-P_2 and α-PRibCP had no effect on AMP inhibition of this enzyme (Fig. 26). β-PRibCP had no effect on Fru-1,6-P_2ase activity at concentrations up to1.2 mM.

DISCUSSION of BIOLOGICAL RESULTS

For 6PF1K, the above results are in contrast to observations made previously (72) and confirm the earlier observations of Rose and Warms (5) and Ishikawa et al. (67) that Rib-1,5-P_2 is a potent activator of 6PF1K. The data from Fig. 23 show that the two Rib-1,5- P_2 analogues do not

activate the enzyme to V_{max}. This phenomenon is especially striking for β-PRibCP which activated the enzyme to only about 30% of its maximal velocity. This kinetic result suggests two mechanistic possibilities: (1) α-or β-PRiBCP may bind to only a fraction of the allosteric sites due to some structural or chemical aspect of the analogues or (2) α– or β-PRibCP may bind to both the substrate and allosteric sites simultaneously. However, based on this and other reports it is unlikely that the first possibility is operative.

Ishikawa et al. (67) have shown that both cyclic and acyclic sugar bisphosphates activate 6PF1K to V_{max} or close to it. This suggests that the orientation of the phosphate groups should not affect binding to the allos-allosteric sites. In this study the differences in acidity between Rib-1,5-P_2 and α–PRiBCP would appear to account for only a moderate decrease in the V_{max} for the activation of 6PF1K by α–PRiBCP versus Rib-1,5-P_2. Therefore, it is also unlikely that these differences in acidity could radically affect binding to the allosteric sites. Furthermore, if Rib-1,5-P_2 and the two analogues bound only to the allosteric sites we

would not expect to see the maximal velocity obtained for each analogue start to decrease as the concentration of activatior was increased.

A second possible mechansim is that these analogues bind simultaneously to the substrate and allosteric sites. It is well documented (68, 70) that an allosteric enzyme can have a ligand which will bind both to the allosteric site and the substrate simultaneously. If this is the case here, we would expect to see a decrease in V_{max} since some fraction of the ligand is inhibiting at the active site while some other fraction is activating at the allosteric site. Furthermore, after it reaches its maximal velocity, we would expect a decrease in velosity since the ligand has saturated the allosteric sites and can now bind exclusively to the substrate site. The β–analogue clearly has a much lower affinity than the α-analogue for either the allosteric or active site of 6PF1K. When it reaches a sufficiently high concentration, it starts to bind the enzyme. However, unlike the α-analogue, the β-analogue has a greater affinity for the active site than for the allosteric site. Hence, it has a much lower maximal velocity. That these compounds will bind to the allosteric site is confirmed by the observation that AMP activation is potentiated by Rib-

1,5-P_2 and the two analogues (Fig. 24). Thus we conclude that α-or β-PRibCP more probably bind simultaneously to the substrate and allosteric sites on 6PF1K.

These results also show that both Rib-1,5-P_2 and α-PRibCP inhibit rat liver Fru-1,6-P_2ase. The data show that the inhibition is independent of Fru-1,6-P_2 concentration (Fig. 25) and that these compounds do notpotentiate AMP inhibition (Fig. 26). These results taken together suggest that Rib-1,5-P_2 and α-PRibCP bind to a site on the enzyme different from either the substrate site or the AMP allosteric site. McClard et al. (71) demonstrated that a mixture of α/β PAraCP inhibited Fru 1,6-P_2ase competitively and that AMP inhibition was potentiated by these compounds. Similar kinetic behavior was observed for both α- and β-anomers of Ara-1,5-P_2 (72). Thus, the trans configuration of the C(2)-C(3) diol group may be a determining factor for binding at the substrate site.

The data presented are consistent with the existence of an additional allosteric site. This claim is not new, however, nor is this claim without a history of controversy. At issue is the physical interpreta-

tion of how Fru-2,6-P_2 inhibits Fru-1,6-P_2ase. Reyes et al. (73) have shown that when pig kidney Fru-1,6-P_2ase is treated with NEM in the presence of AMP it retains catalytic activity and is still inhibited by AMP, but is no longer inhibited by Fru-2,6-P_2. This demonstrates, according to these authors, a distinct allosteric binding site for Fru-2,6-P_2. The K_m for substrate increased by a factor of three after modification, indicating that the cysteine residue affected was distant from the substrate site. Furthermore, they point out that in the native enzyme, Fru-2,6-P_2 inhibition is relieved only at high substrate concentrations and that in the modified enzyme the substrate loses not only this ability, but also the abiltiy to inhibit the enzyme. They concluded that in the native enzyme Fru-2,6-P_2 and high concentrations of substrate bind to this additional allosteric site.

On the other hand, McGrane et al. (74) showed that Fru-2,6-P_2 potentiates inhibition by AMP. Binding studies demonstrated that this effect was due to the ability of Fru-2,6-P_2 to enhance the affinity of the enzyme for AMP. Additionally, both AMP and Fru-2,6-P_2 induce the same uv-difference spectra, suggesting, according to these authors,

that Fru-2,6-P_2 binding at the active site induces a conformational change in the enzyme comparable to that induced by AMP at the allosteric site.

^1H and ^{31}P NMR studies (75) have shown that the distances between enzyme-bound Mg^{2+} and the phosphate group of Fru-6-P and between the 6-phosphate group of Fru-2,6-$P_2$and enzyme-bound Mg^{2+} were the same.

Finally, it has recently been shown by x-ray crystallography that β-Fru-2,6-P_2 binds to the active and perhaps to the AMP site (68). This result must be interpreted with caution. It is quite possible that during the crystallization process ligand binding may not occur at a low affinity binding site (69). In any case it is possible to explain some of the data on chemical modification of the enzyme in terms of the existence of only a catalytic and AMP allosteric site. If one postulates that the enzyme can exist in two different conformations, such that in one form (C) the active site acts as an "allosteric site" and the other form (C') acts as a "substrate site". Further, if Fru-2,6-P_2 only binds to the C form whereas Fru-1,6-P_2 binds to both forms, cysteine modification may prevent the C to C' conver-

sion, hence the enzyme loses its "allosteric site". If so, Fru-2,6-P_2 inhibition would be eliminated and Fru-1,6-P_2 inhibition greatly diminished. The decrease in bindng of metal ions to either structural or catalytic sites could account for the decreased activity (73,76).

Benkovic et al. (77) have shown that 2,5-anhydro-D-glucitol-1,6-P_2 (isosteric to α-Fru-1,6-P_2) and 2,5-anhydro-D-mannitol-1,6-P_2 (isosteric to β-Fru-1,6-P_2) inhibit the enzyme. Moreover, the β-Fru-1,6-P_2-analogue inhibits without potentiating AMP inhibition (78) and at high concentrations (> 1mM) inhibits noncompetitively (77), although Riquelme et al. (78) observed noncompetitive inhibition by the α-analogue and competitive inhibition by the β-analogue. Lastly, x-ray crystal data show that these analogues bind to the substrate site (79).

With regard to the compounds in this study, it is possible to interpret the data based on the assumption that there is not another allosteric site. Both α-Rib-1,5-P_2 and compound α–PRiBCP are structurally similar to AMP. Like AMP, these compounds show noncompetitive inhibition. Therefore it is possible that these two compounds bind to the AMP site. Moreover, the

observation that they do not potentiate AMP inhibition would be expected; Fru-2,6-P_2 potentiates AMP inhibition by binding partially to the AMP site (68). This binding thus induces greater co-operativity for AMP binding.

If Rib-1,5-P_2 and analogue α–PRibCP bind exclusively to the AMP site they cannot induce greater co-operativity for AMP binding, hence, no potentiation would be expected. β-PRibCP does not inhibit at concentrations up to 1.2 mM. The greater polarity of its methylene phosphonate group relative to that of the adenosine group of AMP may be sufficient to repel β-PRibCP from binding.

It is also possible that these ribose compounds can bind to the substrate site. Rib-1,5-P_2 and α-PRibCP behave kinetically like 2,5-anhydro-D-mannitol-P_2; they inhibit noncompetitively and do not potentiate AMP inhibition. They may bind to the one form of the enzyme (as discussed above) and prevent conversion from one form to the other. This may account for the noncompetitive inhibition.

CONCLUSIONS

Two conformational analogues of Rib-1,5-P_2 have been prepared. These analogues were made by replacing the phosphate group on C(1) of Rib-1,5-P_2

with a methylene phosphonate group. Both the α- and β-anomers were isolated. The anomeric configuration of each isomer was determined by literature precedent. In addition, an analysis of mass spectral data provided confirmatory evidence for the anomeric assignments made.

Kinetic tests, performed on 6PF1K, demonstrated that Rib-1,5-P_2 and both α- and β-PRibCP are activators, and that at sufficiently high enough concentrations all three compounds bound simultaneously to both the active site and the allosteric site.

Both Rib-1,5-P_2 and α-PRibCP inhibited Fru-1,6-P_2ase with approximately the same K_I value (87 μM). Neither of these two compounds potentiated AMP inhibition of the enzyme. β-PRibCP did not inhibit the enzyme nor did it potentiate AMP inhibition.

It is possible to interpret the results of the 6PF1K studies as suggesting that at high enough concentrations Rib-1,5-P2, α-PRibCP, and β-PRibCP bind simultaneously to the active site and the allosteric site.

It is also possible to interpret the results of the Fru-1,6-P2 studies

as indicating the existence of a third binding site, although other explanations are possible.

In conclusion, the two Rib-1,5-P_2 analogues affect the activity of 6PF1K and Fru-1,6-P_2ase.

TABLE 1.

SUMMARY OF KINETIC RESULTS FOR 6PF1K

Activation of 6PF1K

ACTIVATOR	K_A (μM)	$\%V_{max}$
Fru-2,6-P_2	0.086 ± 0.002	100*
Rib-1,5-P_2	3.5 ± 0.2	100
α-PRibCP	6.5 ± 0.2	80
β-PRibCP	> 52 ± 1.3	30

Fru-6-P concentration was 0.25 mM.

*V_{max} was established by using 4 mM Fru-6-P and 10 μM Fru-2,6-P_2 in the assay mixture.

Potentiation of AMP Activation of 6PF1K

ACTIVATOR	K_m (μM)	%Vmax
AMP	10.0 ± 0.1	78
AMP + Rib-1,5-P_2	2.5 ± 0.1	90
AMP + α-PRibCP	2.5 ± 0.2	83
AMP + β-PRibCP	5.0 ± 0.1	75

Fru-6-P concentration was 0.25 mM.

TABLE 2.

SUMMARY OF KINETIC RESULTS FOR Fru-1,6-P_2ase

Inhibition of Fru-1,6-P_2ase

INHIBITOR	K_I(μM)	%Maximal Decrease in Activity
At 5 μM Fru-1,6-P_2		
Rib-1,5-P_2	87	50
α-PRibCP	87	50
At 20 μM Fru-1,6-P_2		
Rib-1,5-P_2	87	50
α-PRibCP	87	50
β-PRibCP	—	—

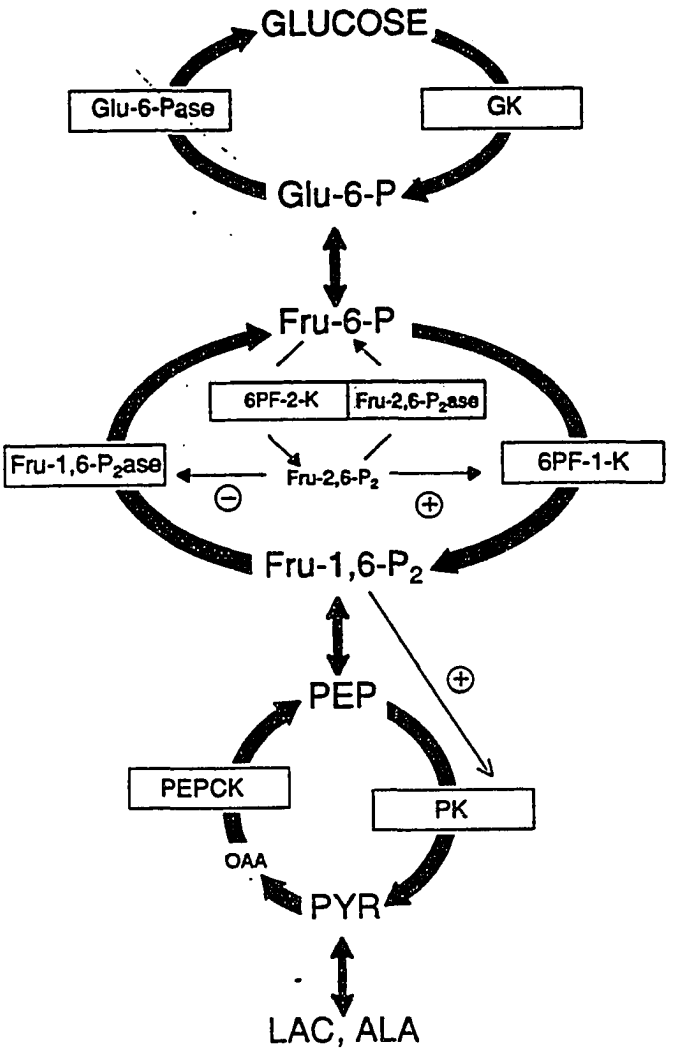
Fru-1,6-P_2 concentration was 5 μM.

Potentiation AMP Inhibition of Fru-1,6-P_2ase

INHIBITOR	K_I(μM)	Shape of Curve
AMP	30	Sigmoidal
AMP + Fru-2,6-P_2 (2 μM)	22	Hyperbolic
AMP + Fru-2,6-P_2 (20 μM)	5	Hyperbolic
AMP + Rib-1,5-P_2 (60 μM)	30	Sigmoidal
AMP + α-PRibCP (60 μM)	30	Sigmoidal

Fru-1,6-P_2 concentration was 5 μM.

REGULATION OF SUBSTRATE CYCLE ENZYMES



Substrate cycles in the glycolytic/gluconeogenic pathway. The enzymes of the three hepatic substrate cycles, which are subject to short-term and long-term regulation by hormones, are L-type pyruvate kinase (PK), phosphoenolpyruvate carboxykinase (PEPCK), fructose 1,6-bisphosphatase (Fru-1,6-P_2ase), 6-phosphofructo-1-kinase (6-PF-1-K), 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase (6-PF-2-K/Fru 2,6-P_2ase), glucokinase (GK), and glucose 6-phosphatase (Glu-6-Pase). Fructose-2,6-bisphosphate (Fru-2,6-P_2) is an activator of 6-PF-1-K and an inhbitor of Fru-1,6-P_2ase.

Fig. 1 Taken from Pilkis and Granner (48)

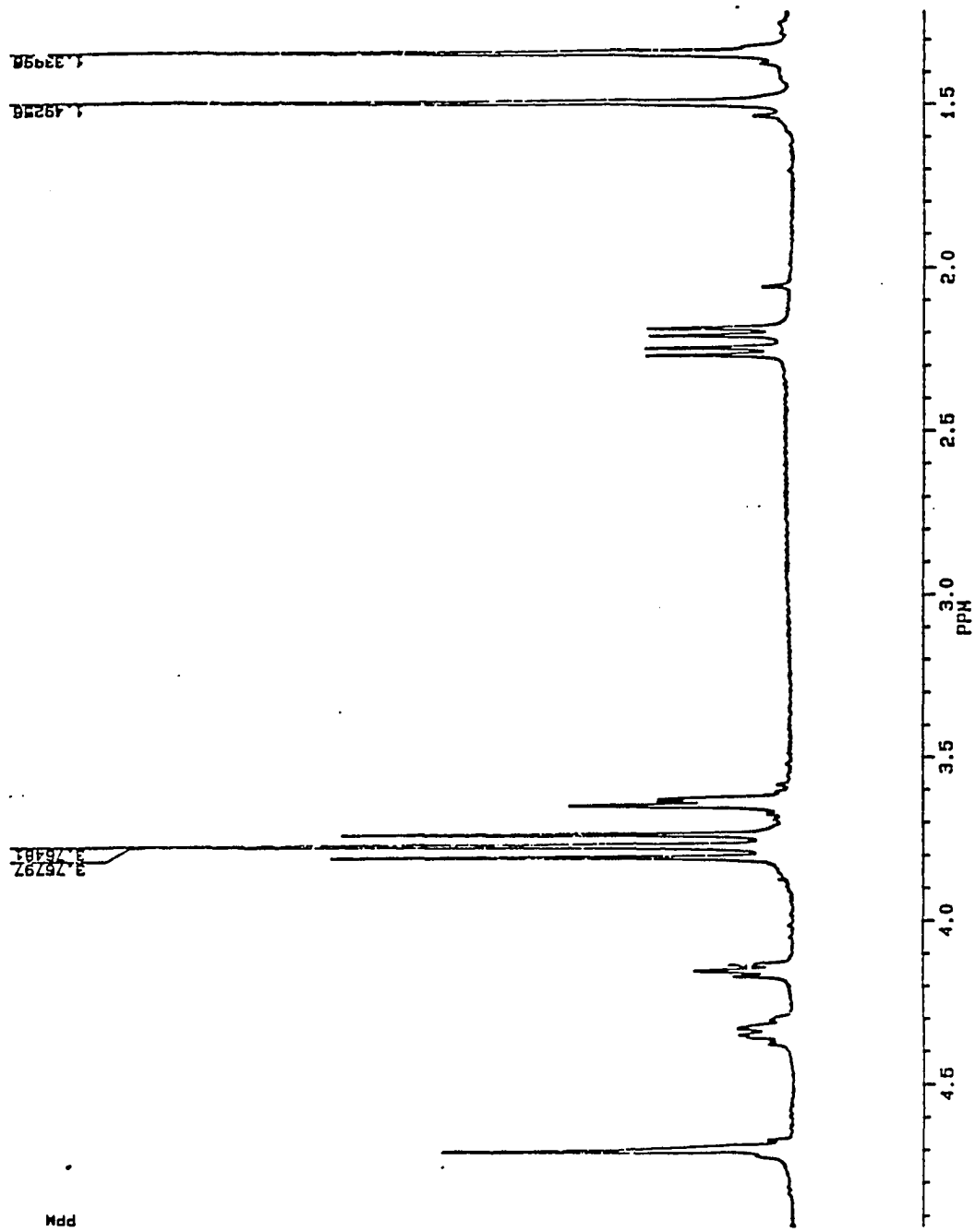


Figure 2: ^{1}H NMR spectrum of 5a

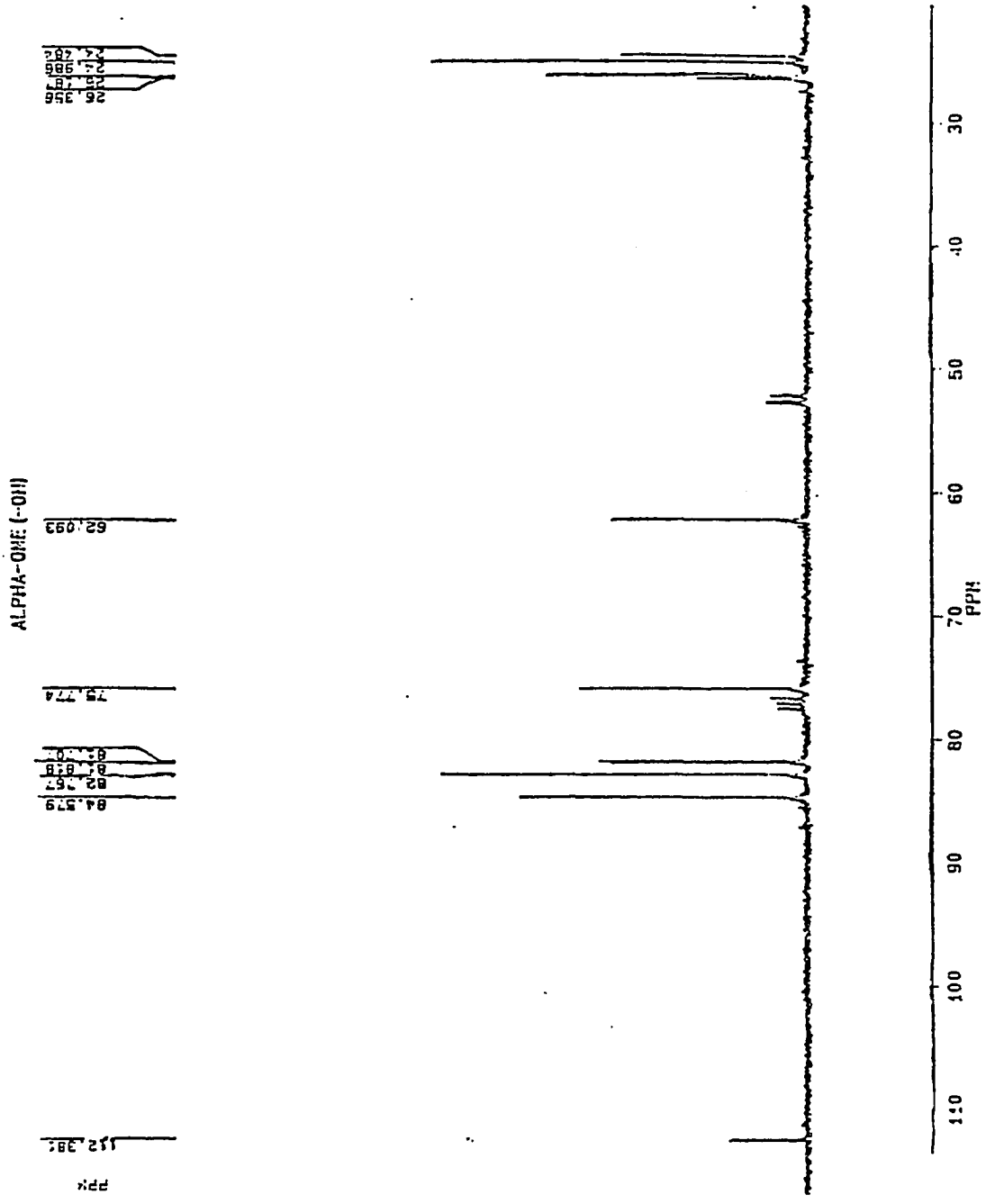


Figure 3: ^{13}C NMR spectrum of 5a

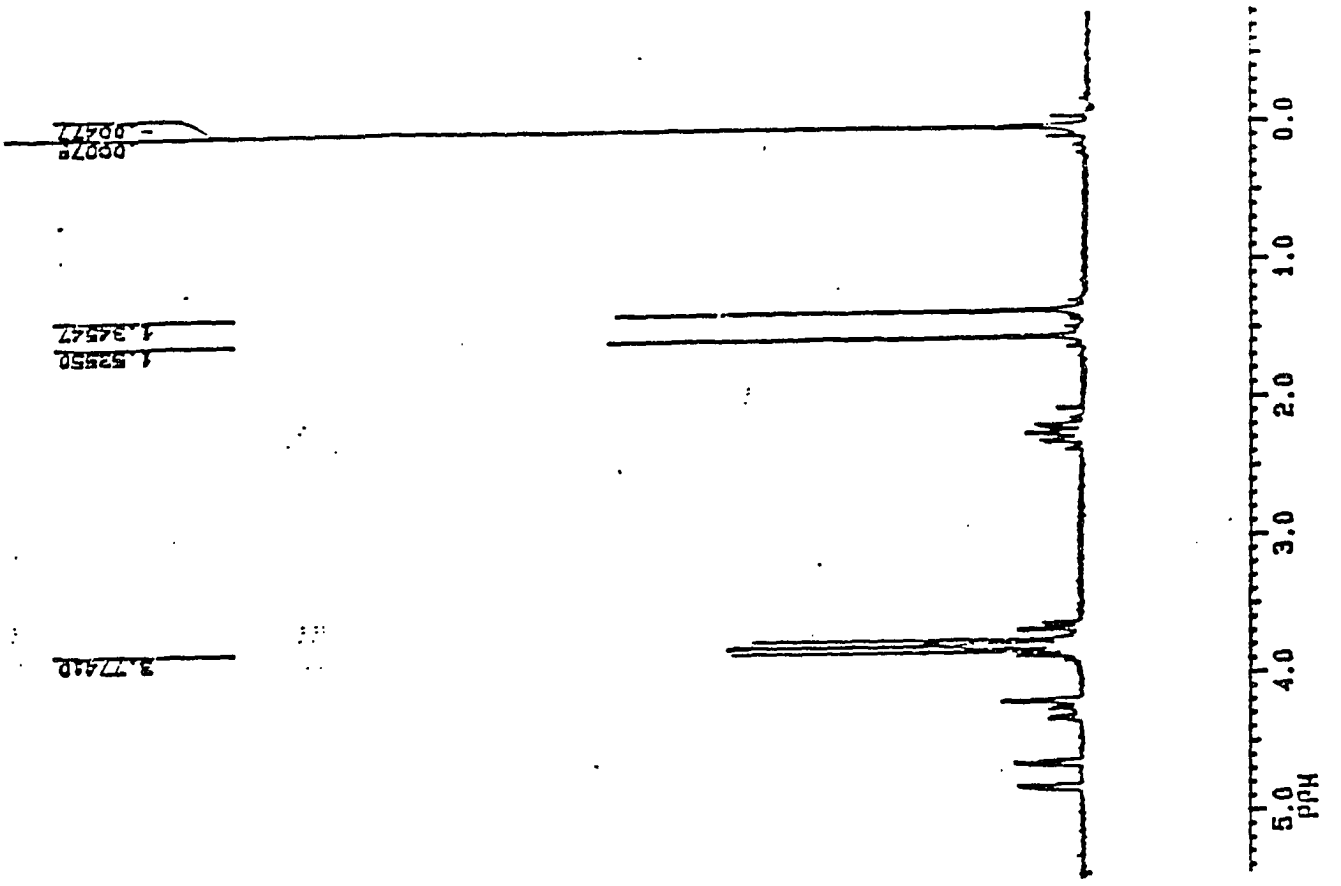


Figure 4: ^{1}H NMR spectrum of 5b

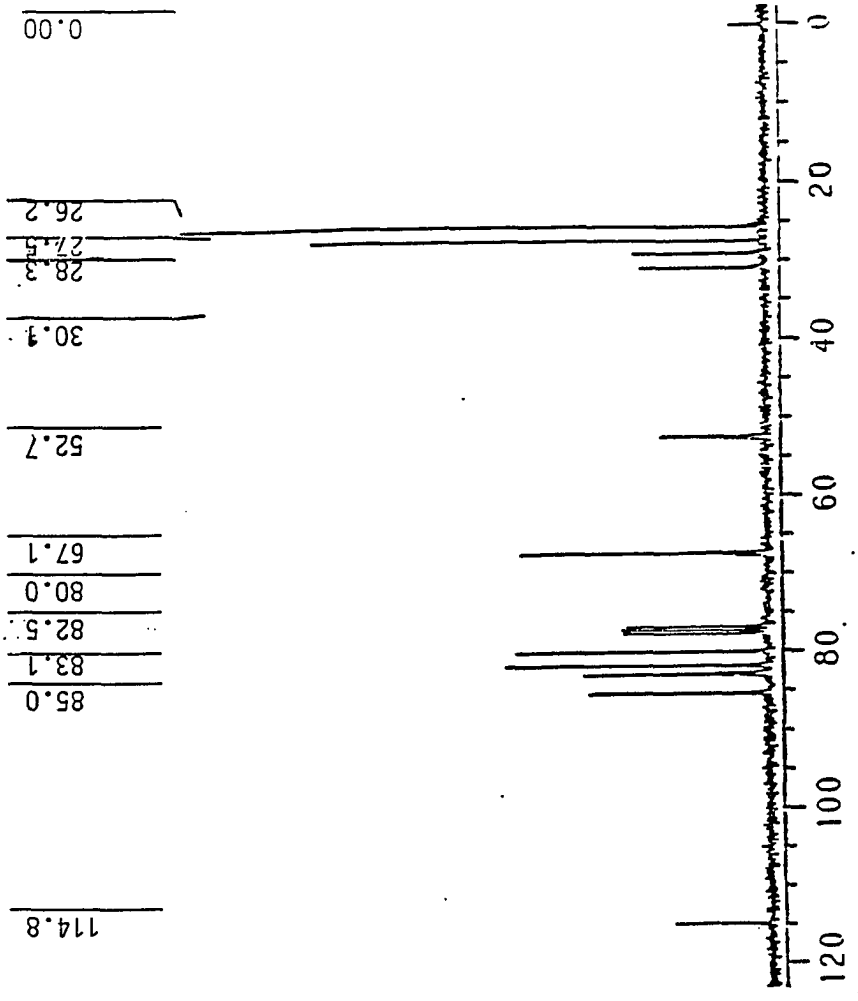


Figure 5: ^{13}C NMR spectrum of 5b

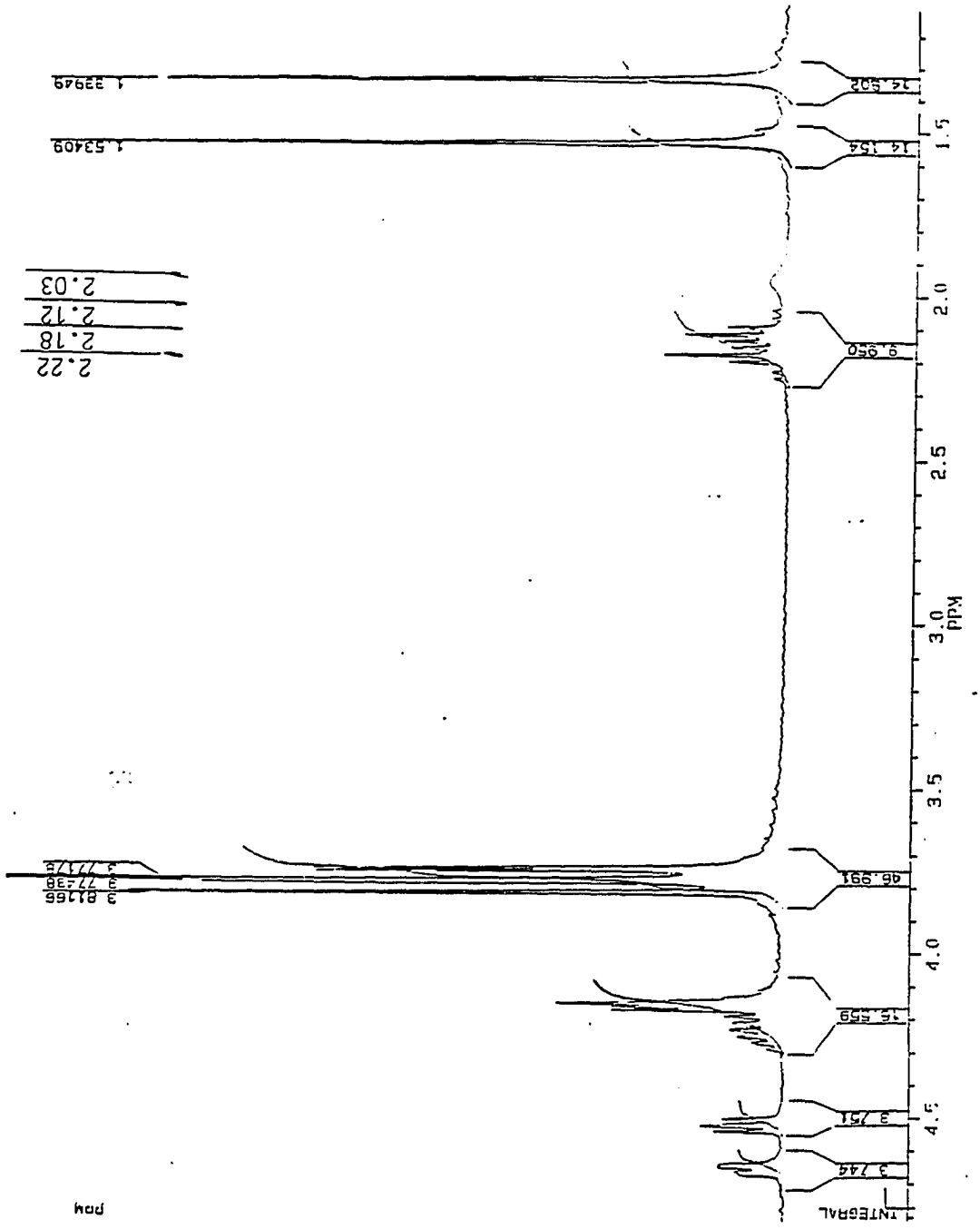


Figure 10: ^{1}H NMR spectrum of 6b

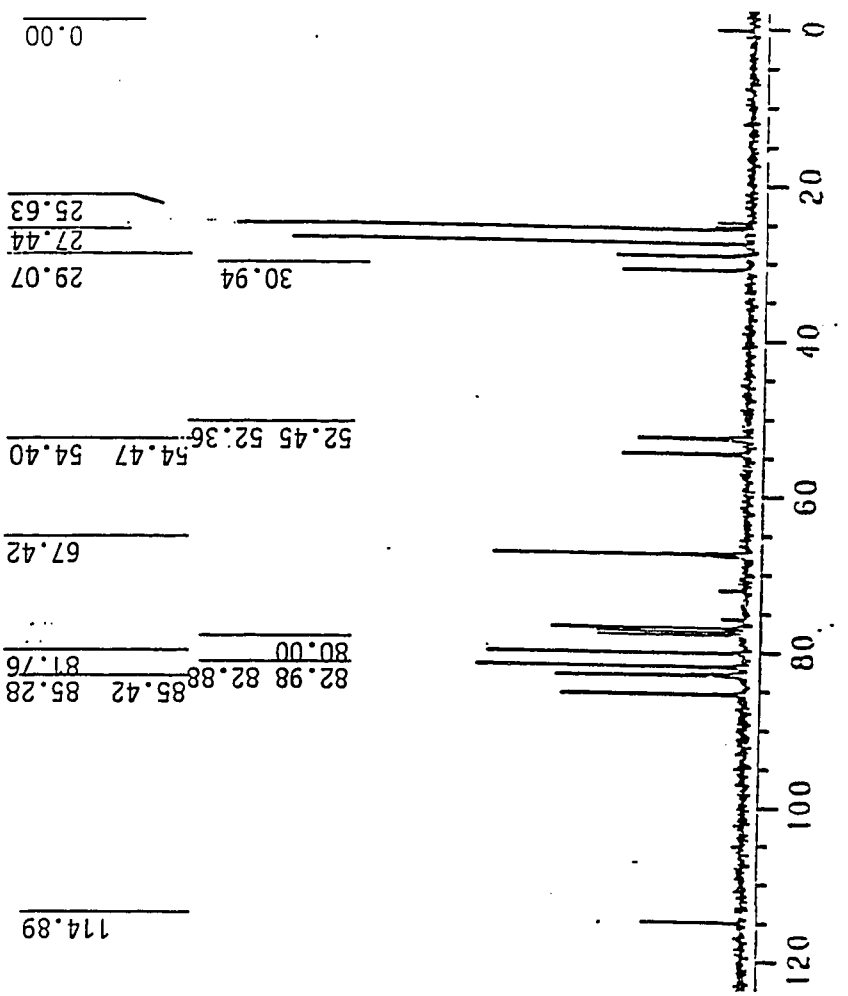


Figure 7: ^{13}C NMR spectrum of 6a

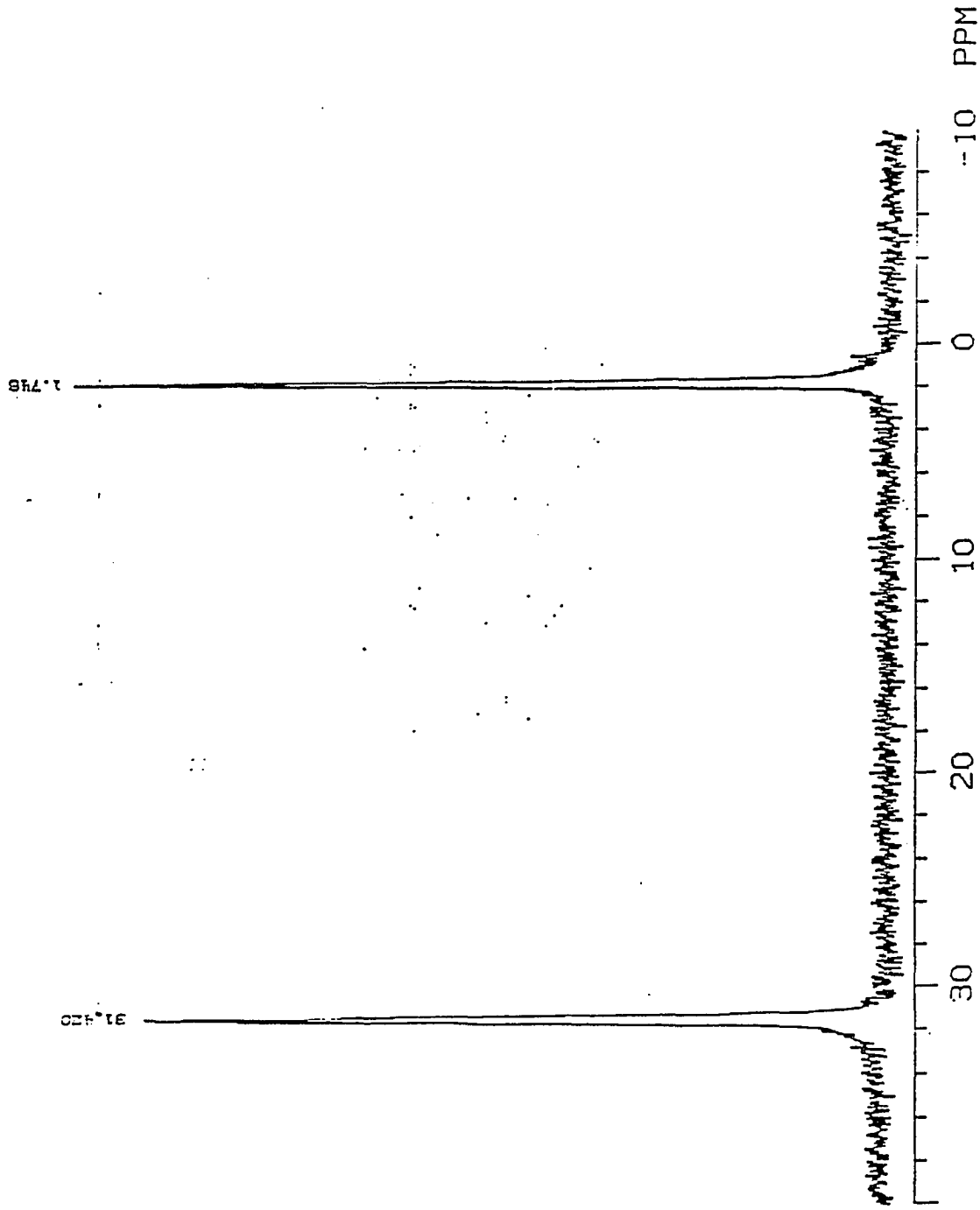


Figure 8: ^{31}P NMR spectrum of 6a

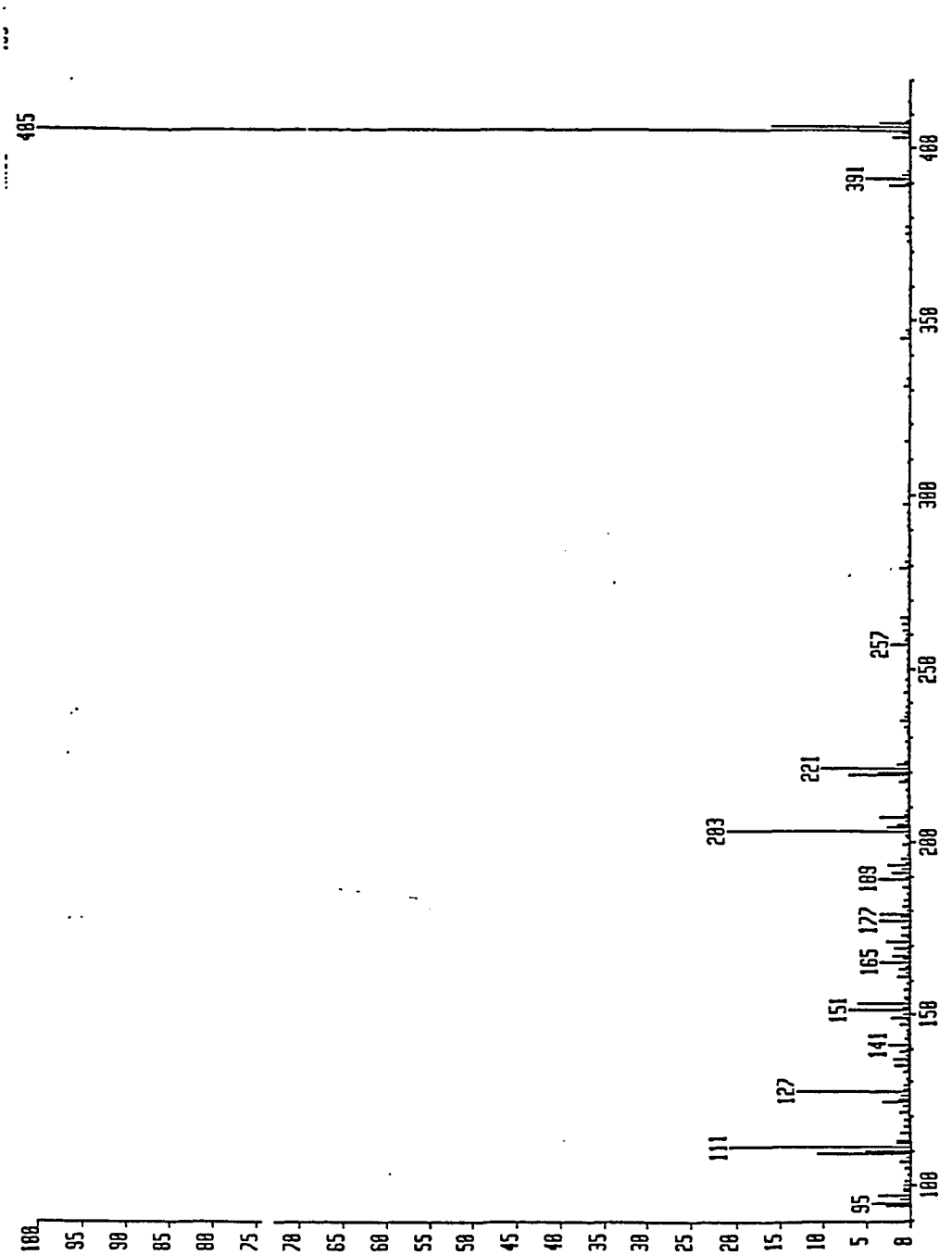


Figure 9: Mass spectum (low resolution) of 6a

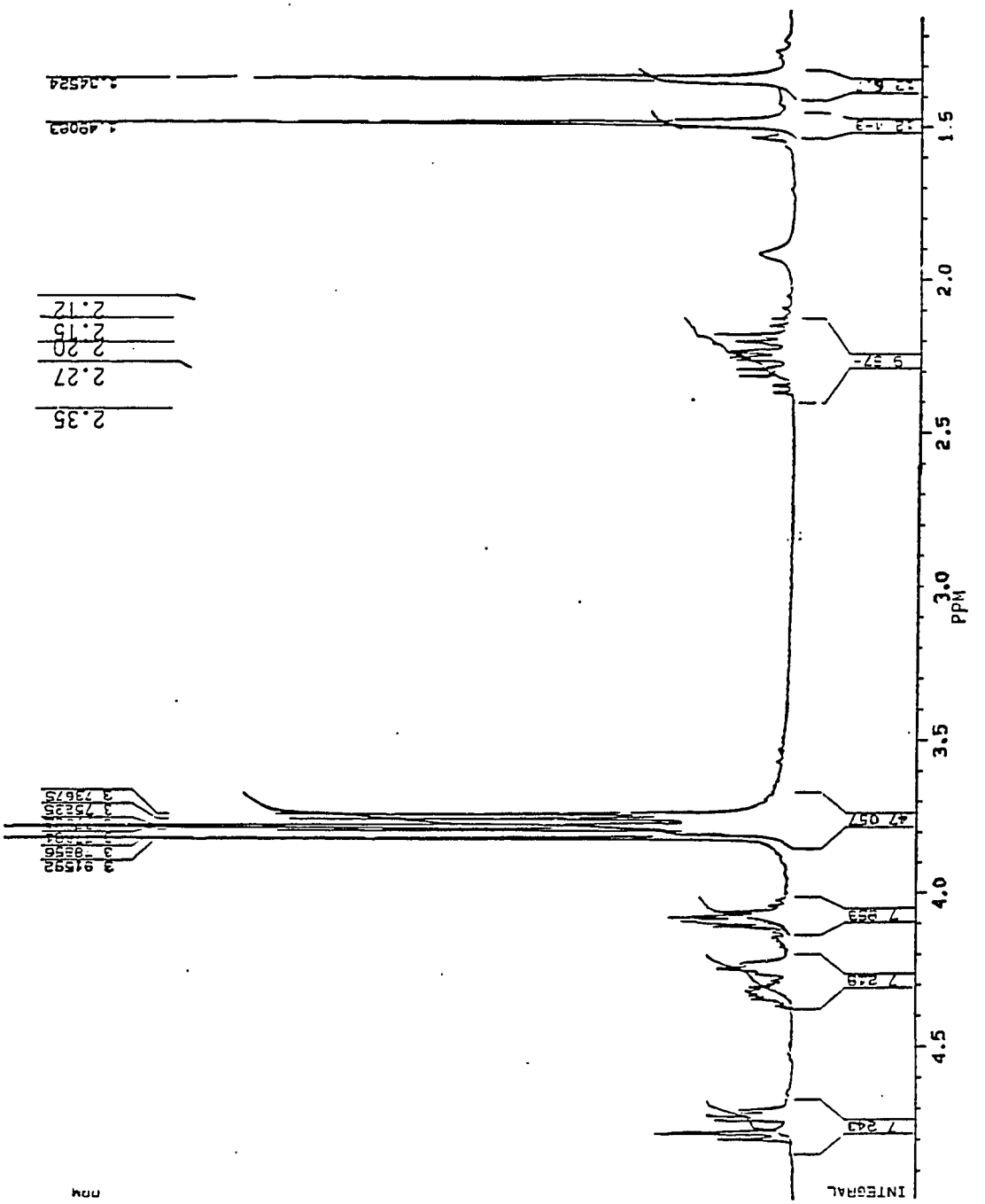
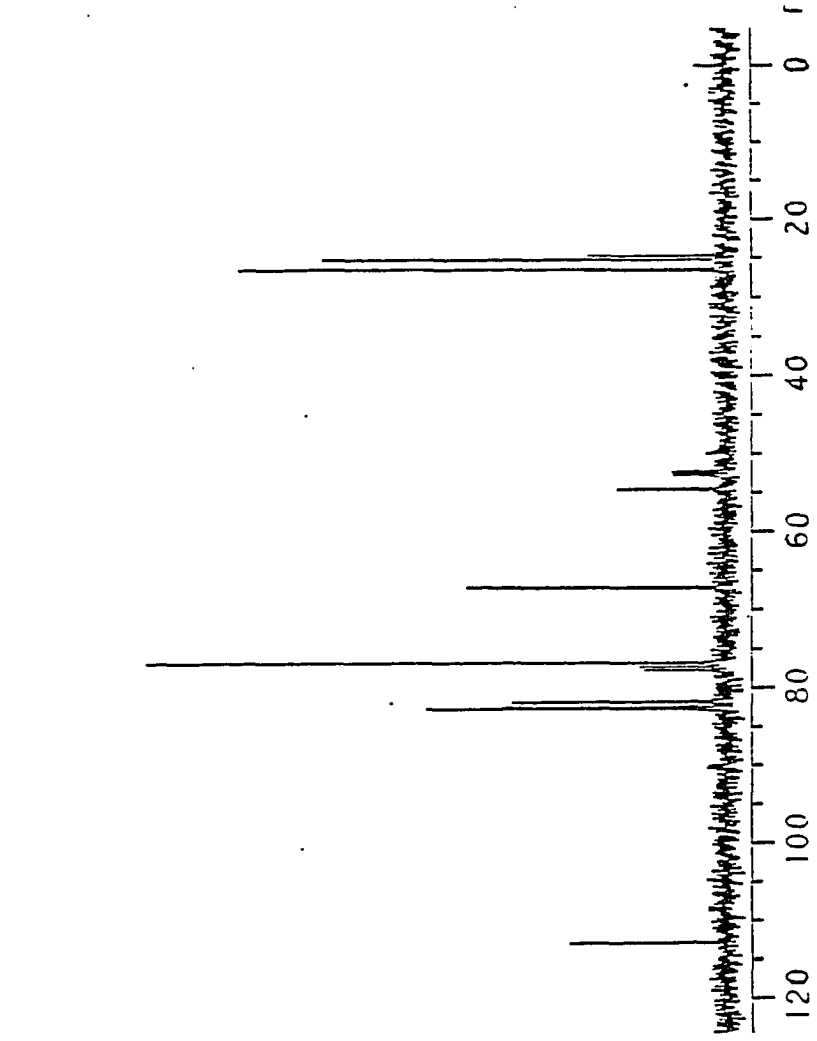


Figure 6: ^{1}H NMR spectrum of 6a



PEAK LISTING

#	HT	PPM
1	24	24.55
2	73	25.04
3	[illegible]	26.26
4	25	26.43
5	9	52.16
6	9	52.54
7	15	54.31
8	19	54.39
9	46	67.01
10	42	67.69
11	100	76.62
12	19	76.68
13	15	77.12
14	[illegible]	77.19
15	14	77.54
16	[illegible]	77.60
17	[illegible]	81.63
18	[illegible]	81.69
19	[illegible]	82.34
20	[illegible]	92.40
21	[illegible]	92.56
22	26	112.80

Figure 11: ^{13}C NMR spectrum of 6b

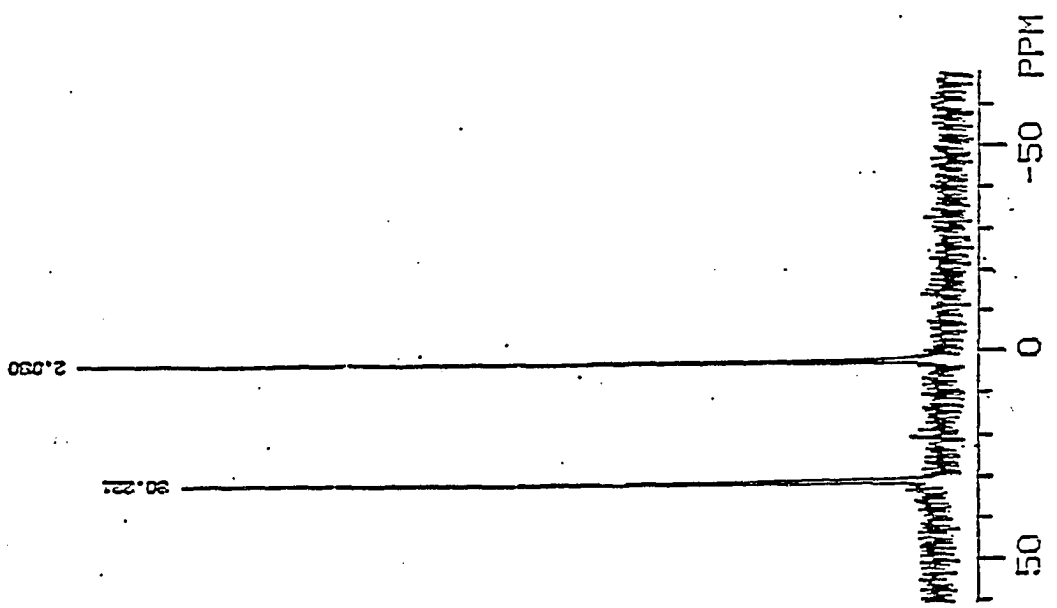


Figure 12: ^{13}P NMR spectrum of 6b

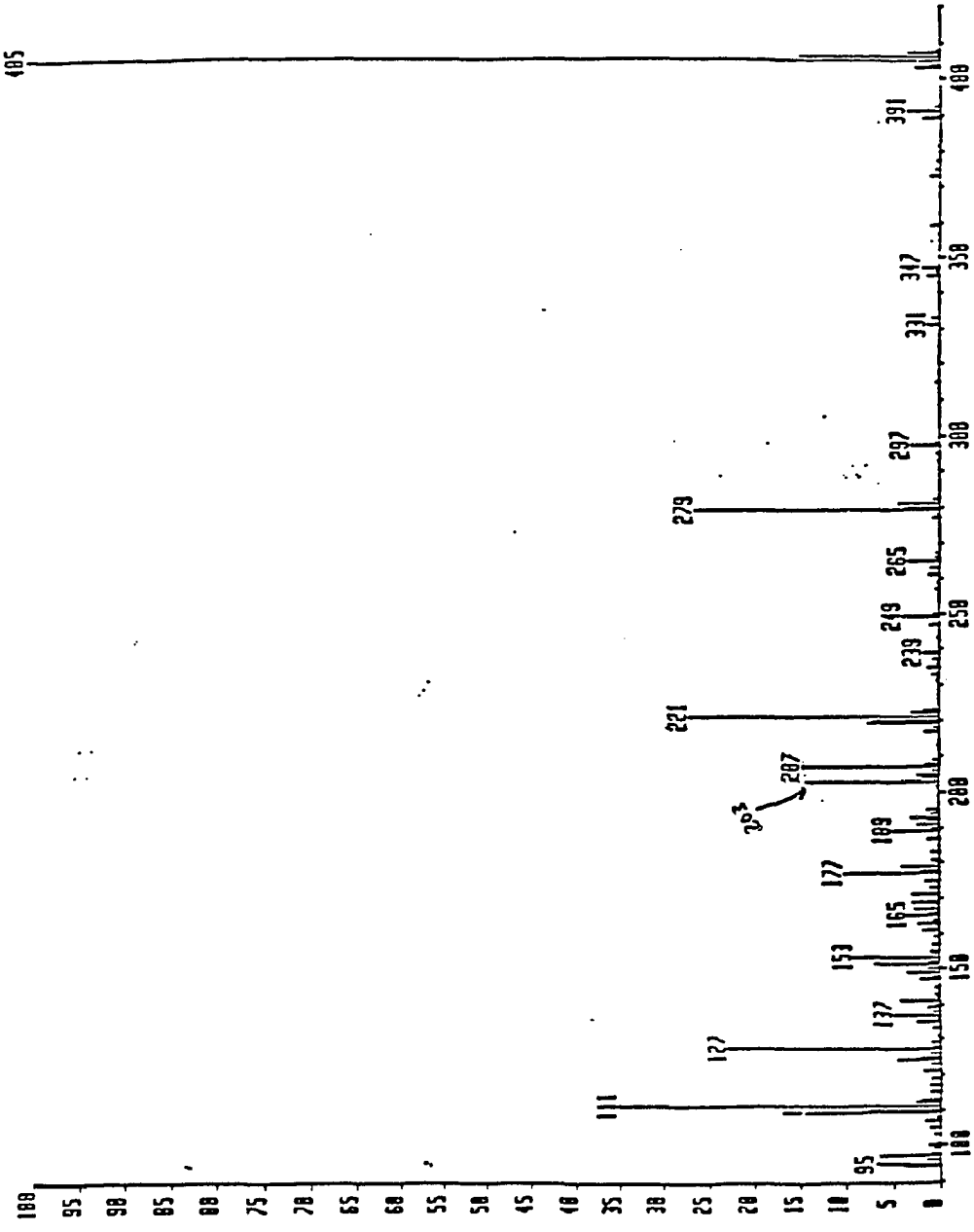


Figure 13: Mass spectrum (low resolution) of 6b

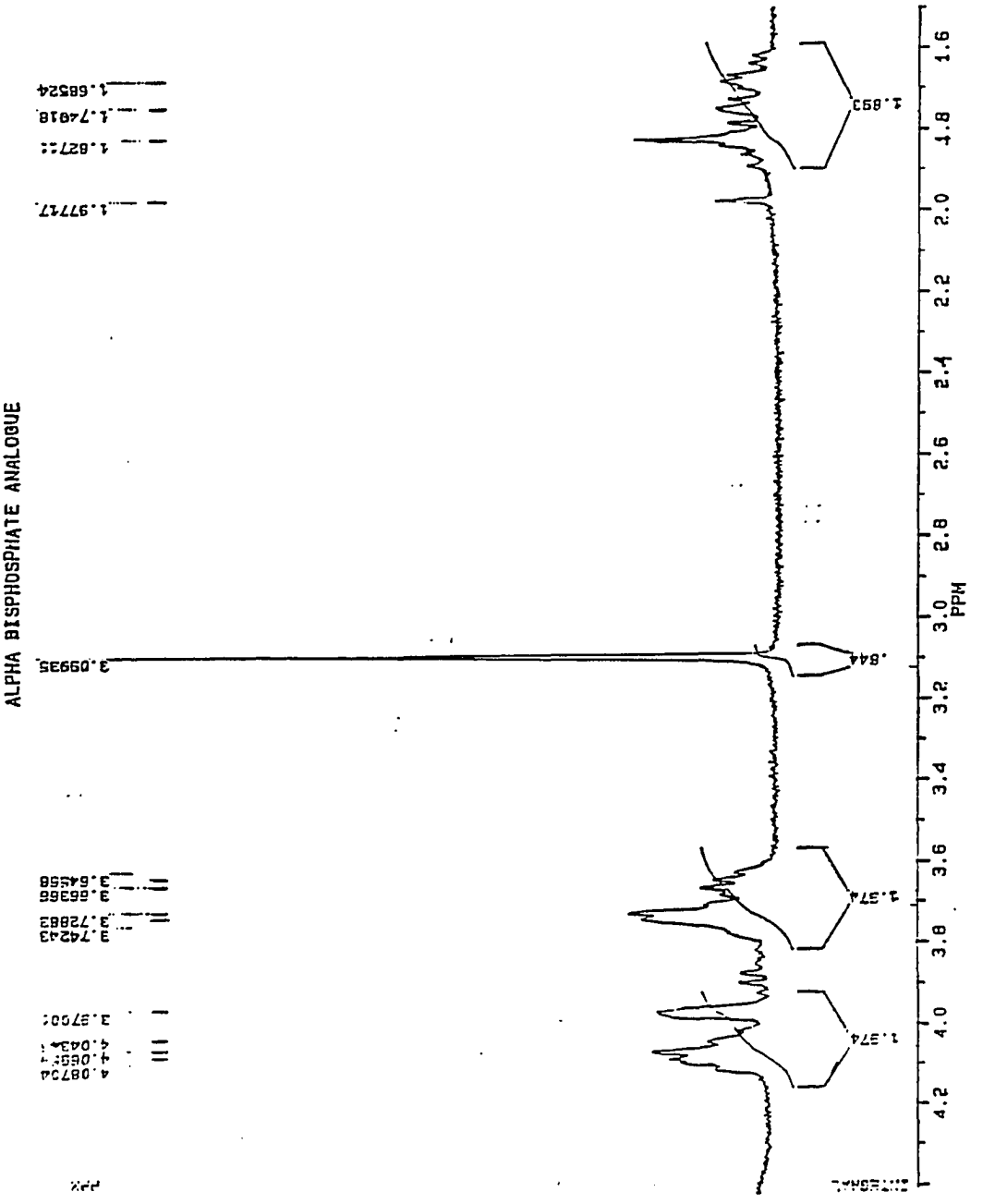


Figure 14: ^{1}H NMR spectrum of 7a

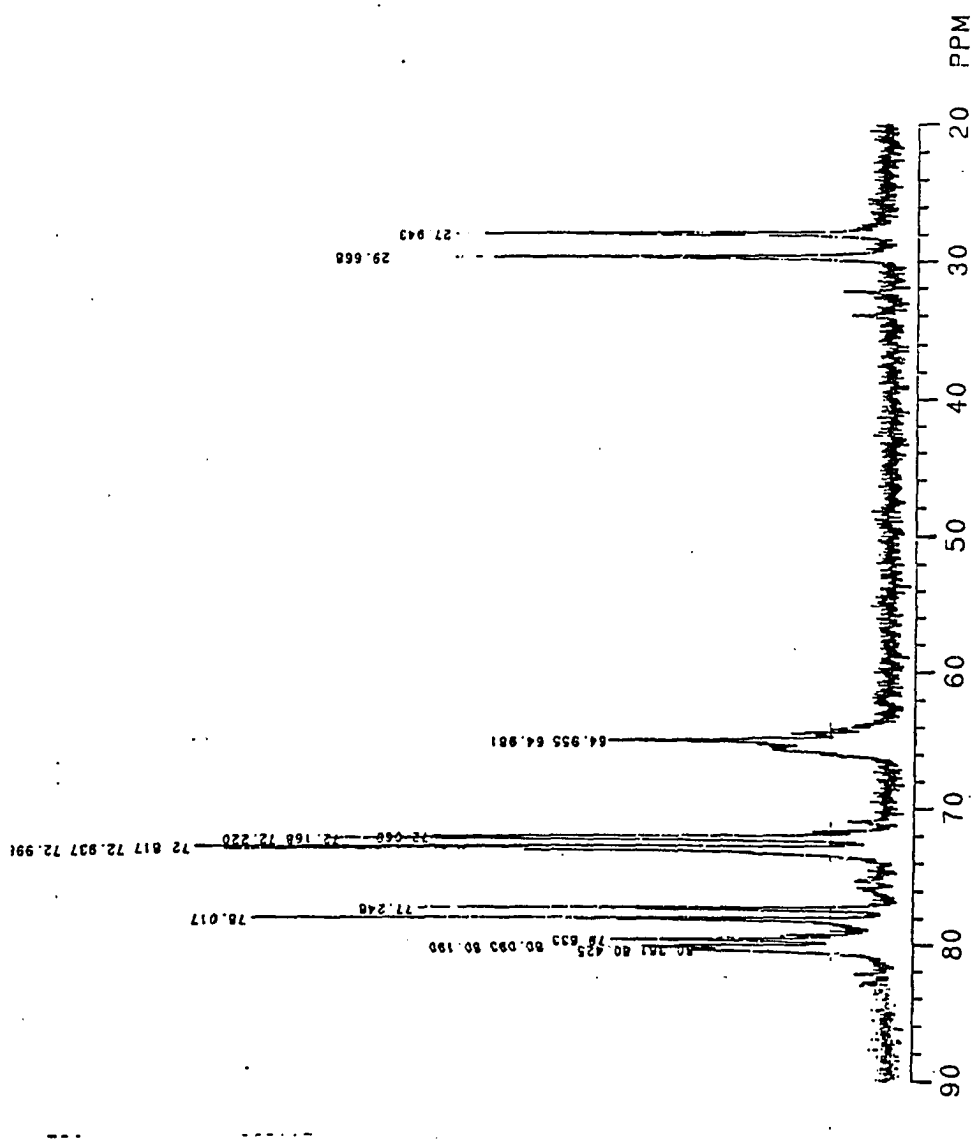


Figure 15: ^{13}C NMR spectrum of 7a

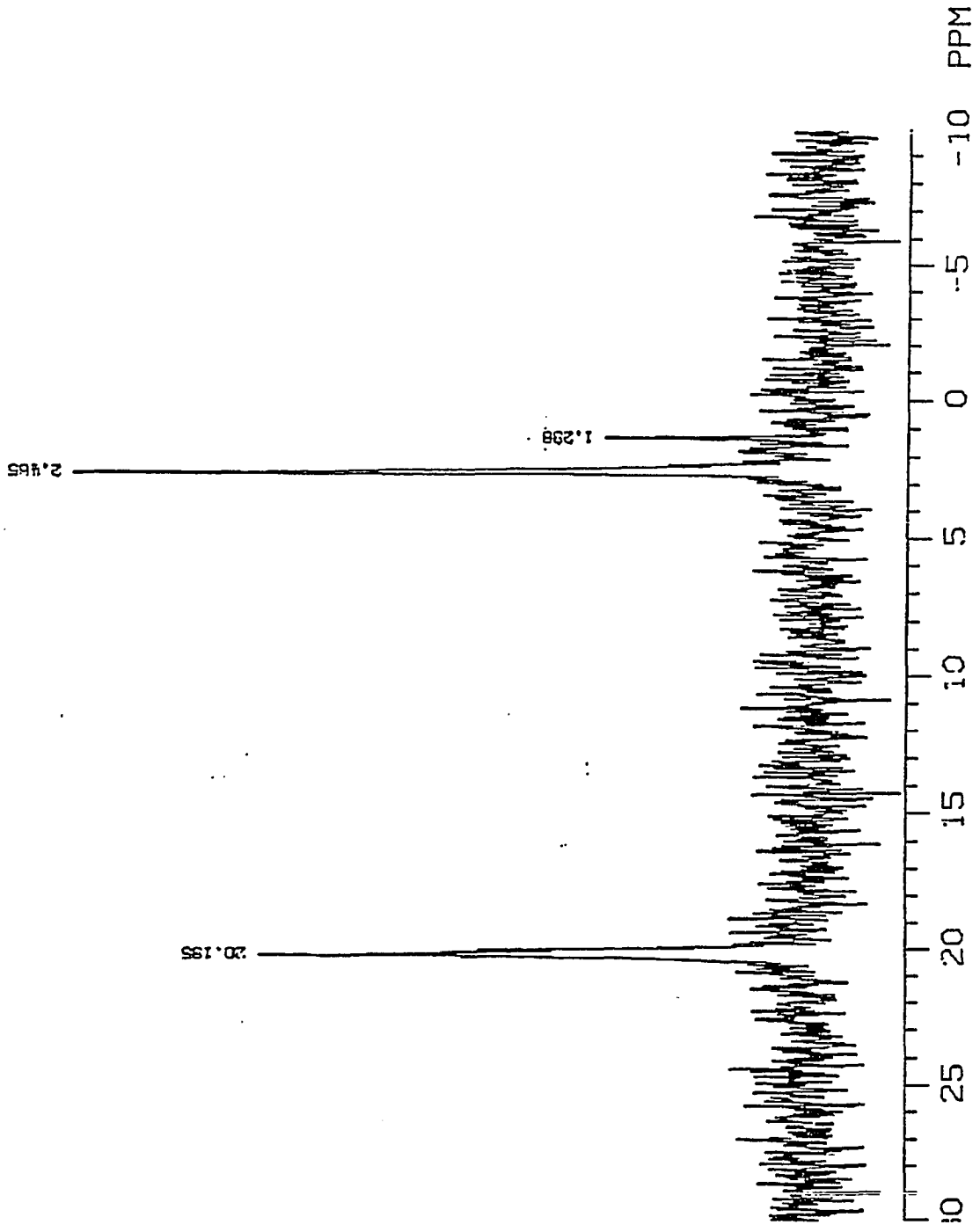


Figure 16: ^{31}P NMR spectrum of 7a

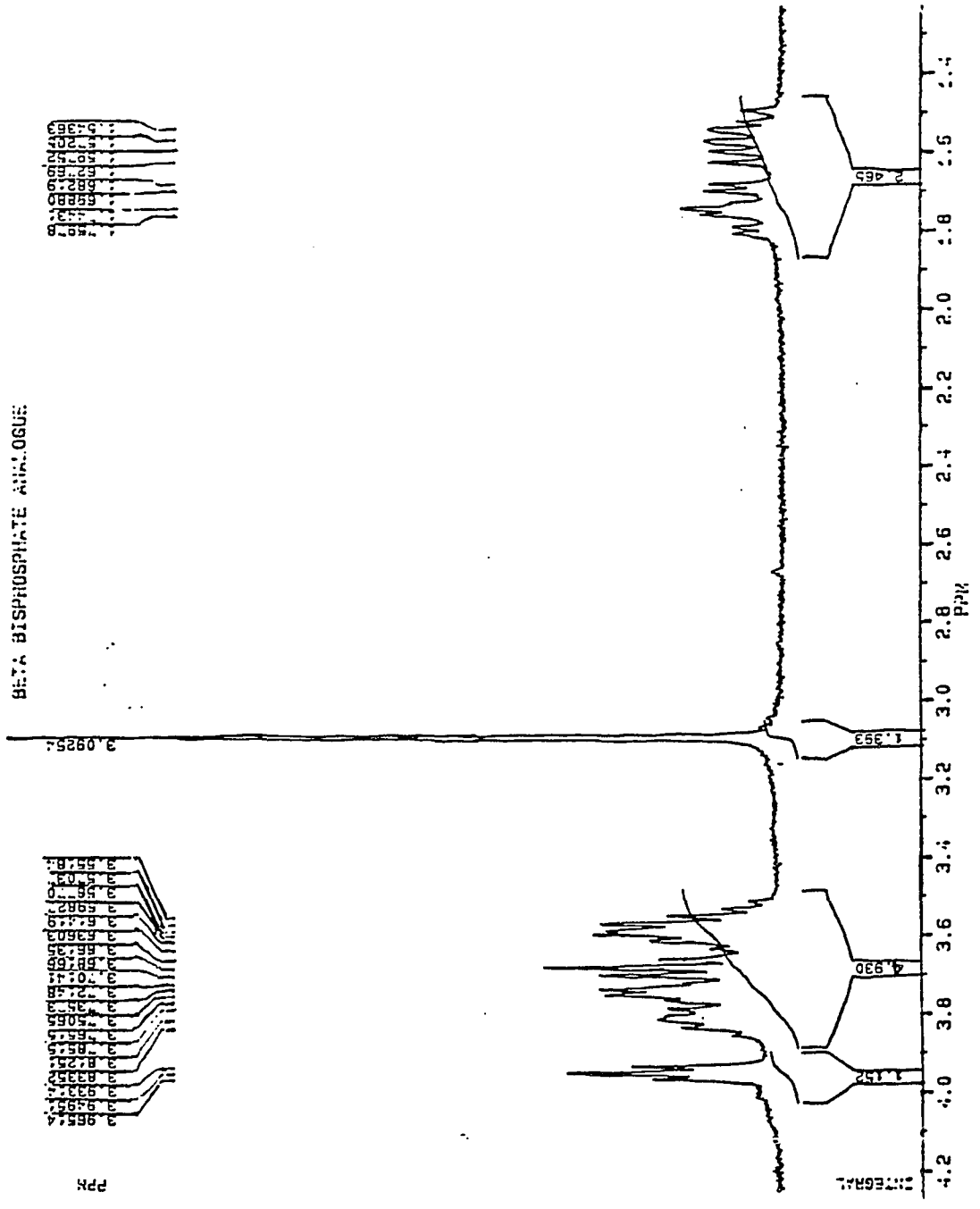


Figure 17: ^{1}H NMR spectrum of 7b

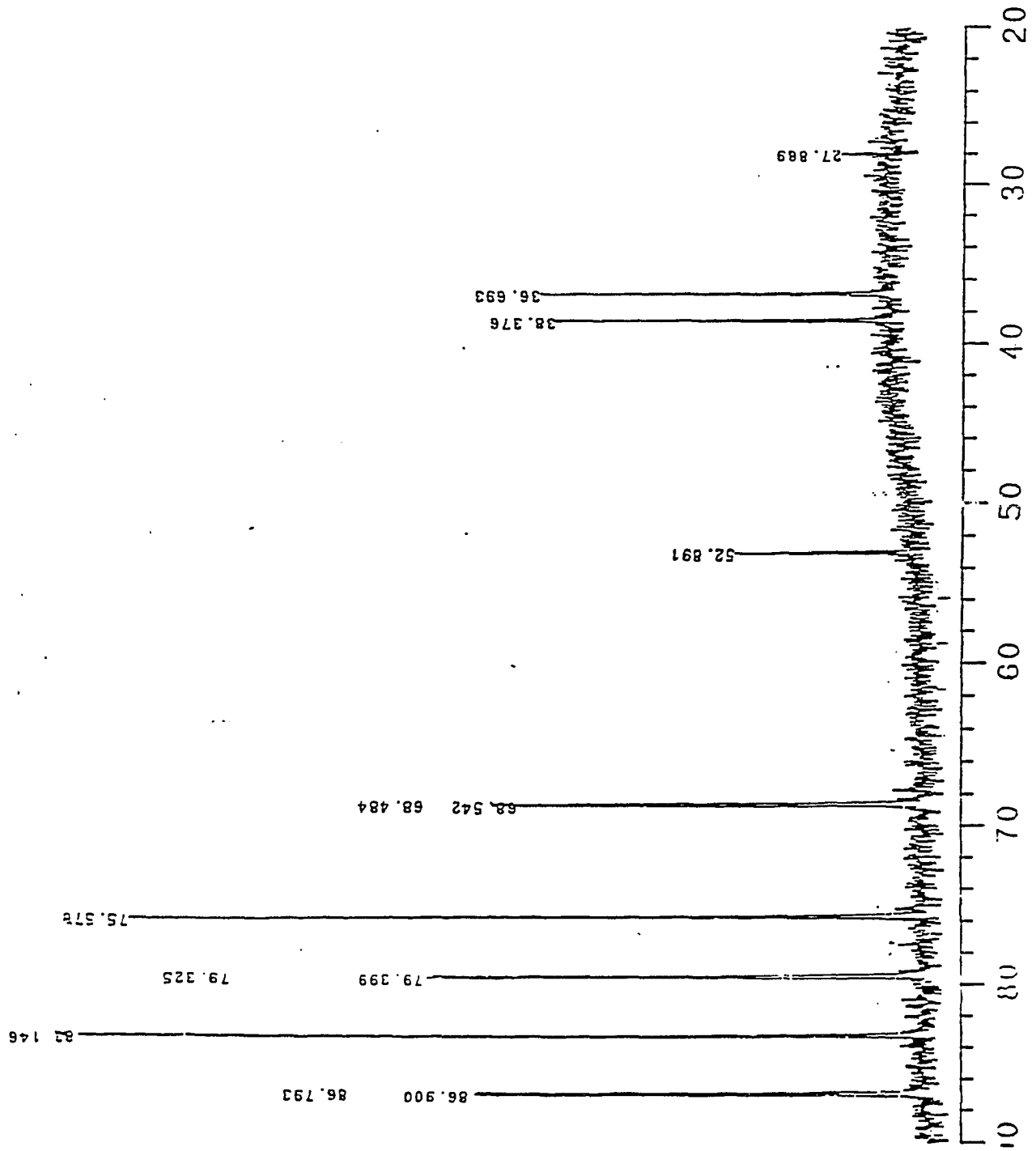


Figure 18: ^{13}C NMR spectrum of 7b

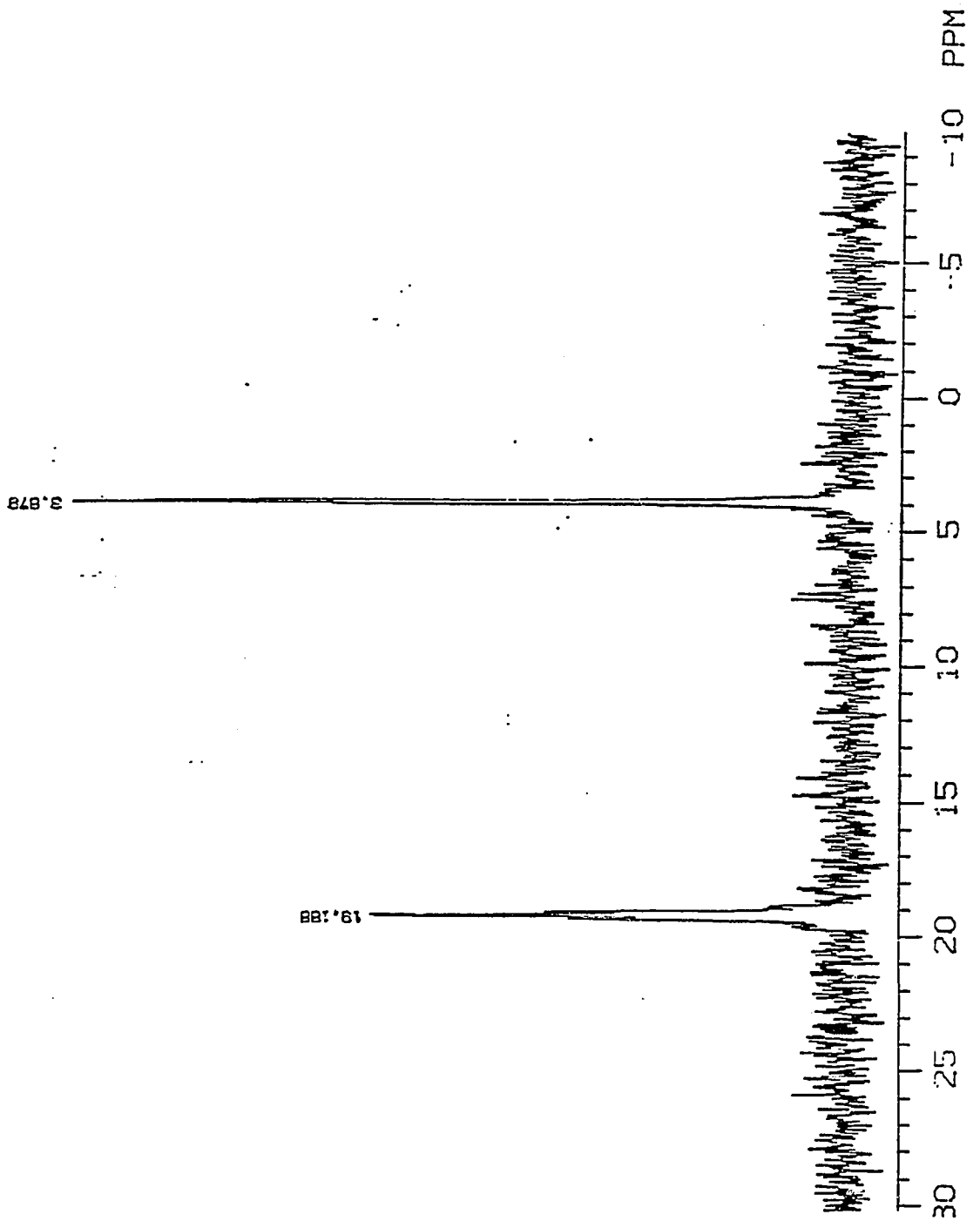


Figure 19: ^{31}P NMR spectrum of 7b

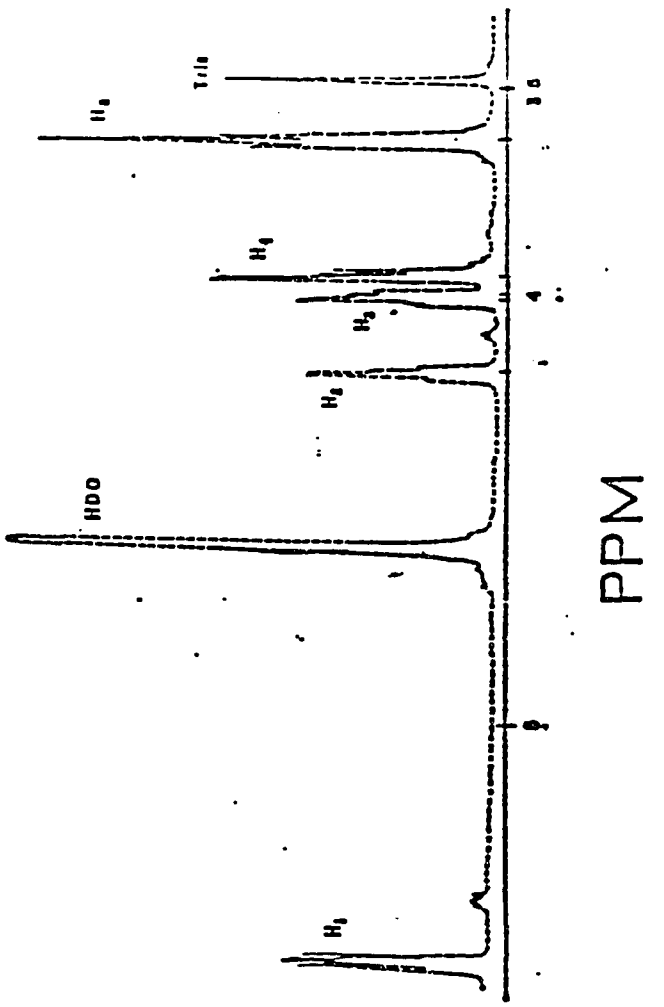


Figure 20: ^{1}H NMR spectrum of PRibPP; ring protons H_2, H_3, and H_4 resemble the splitting pattern of 7a (Fig. 14) more so than the splitting pattern of 7b (Fig. 17).

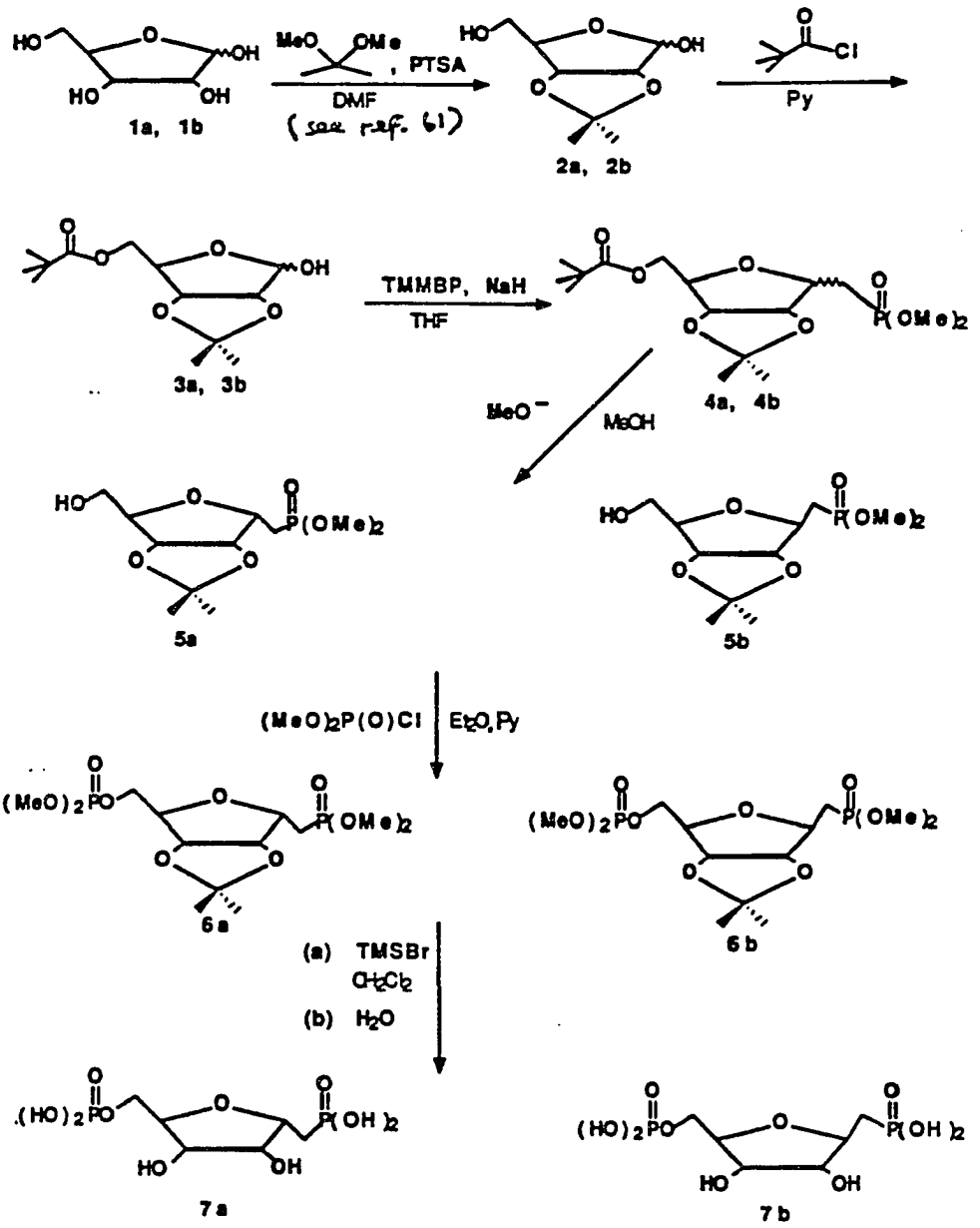


Fig. 21 Synthetic Scheme

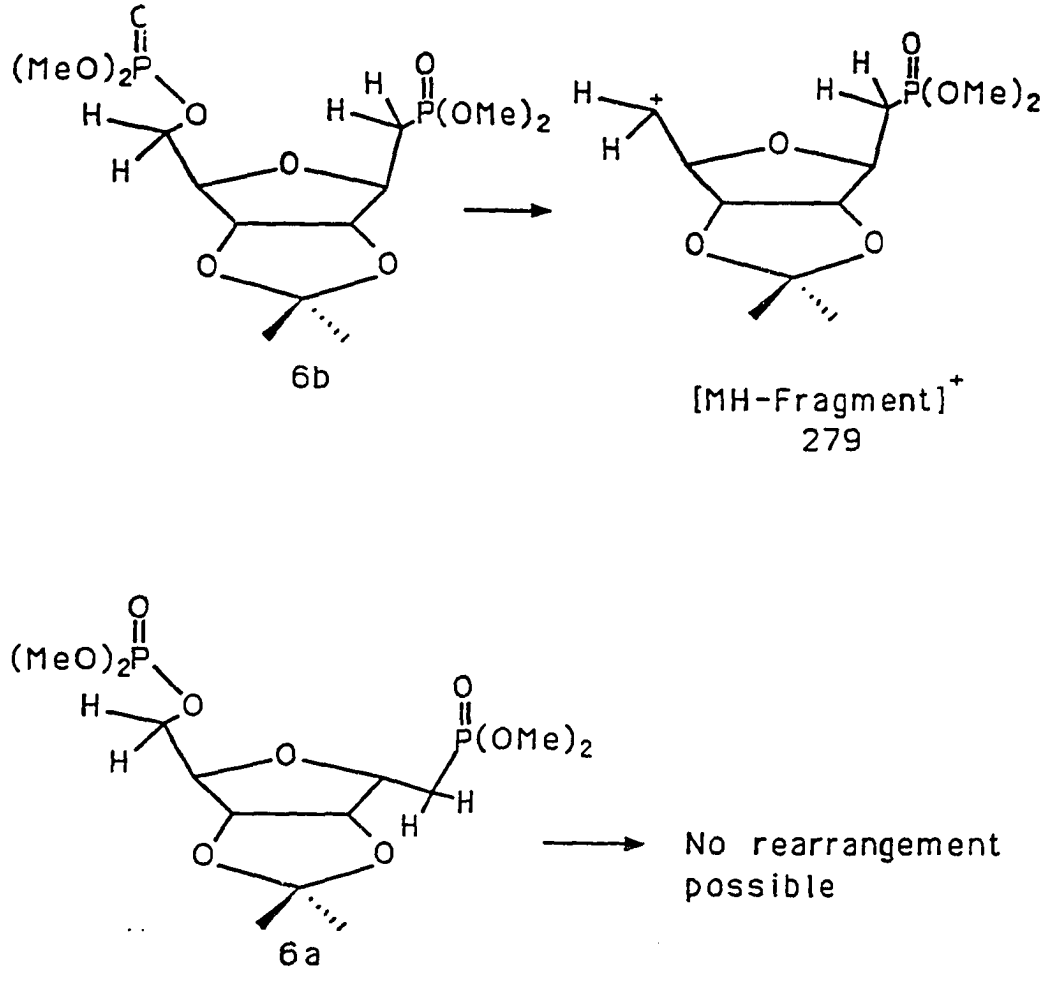


Fig. 22. Possible mechanism to explain fission fragments of 6a and 6b: proton on C(1) atom can jump to C(6) oxygen atom; resulting fragments may be more stable than parent ion. 6a does not have correct orientation for rearrangement.

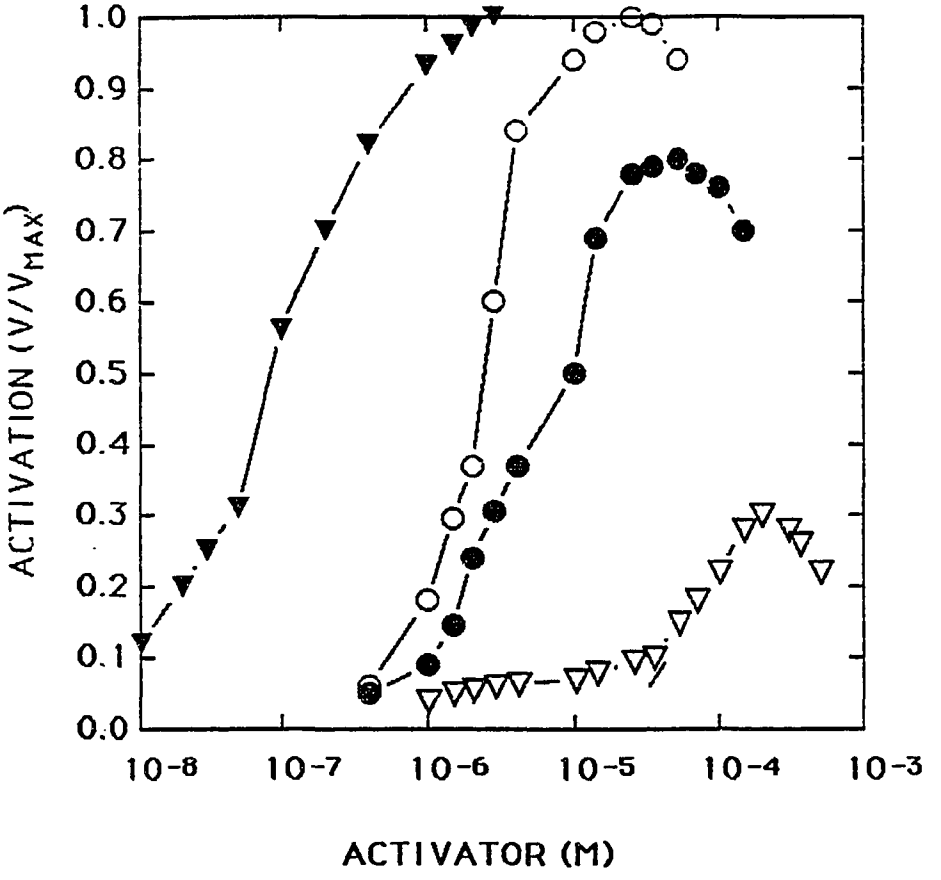


Figure 23: Activation of 6PF1K by Fru-2,6-P_2 (▼), Rib-1,5-P_2 (○), α-PRibCP(●), β-PRibCP (▽). Fru-6-P concentration is 0.25 M.

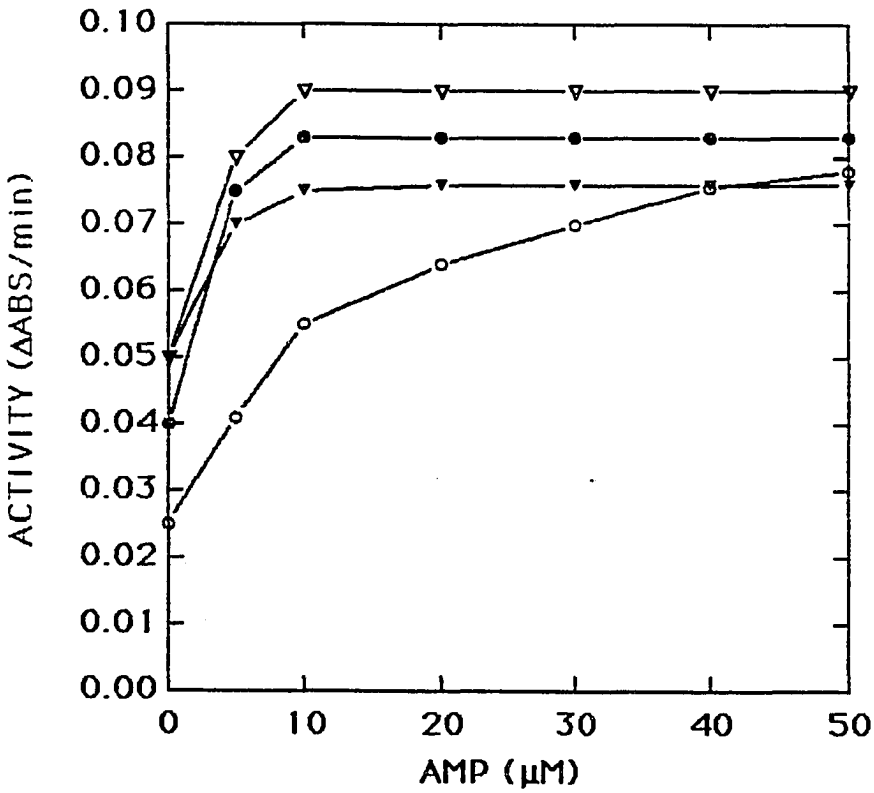


Figure 24: Potentiation of AMP Activation of 6PF1K. AMP alone (○), with 1.5 μM Rib-1,5-P_2 (▽), 3.5 μM α-PRibCP (●) and 50 μM β-PRibCP (▼). Fru-6-P concentration was 0.25 M.

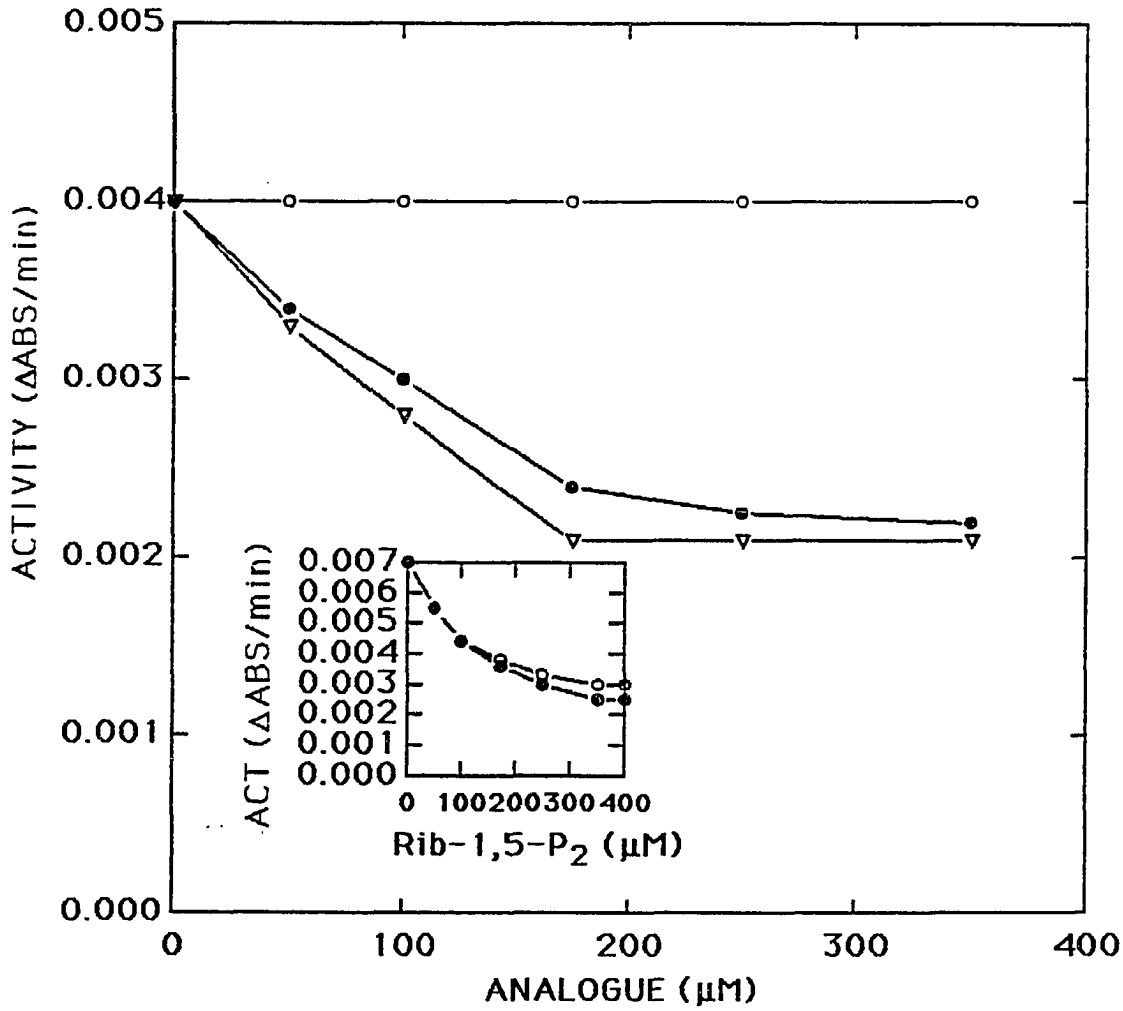


Figure 25: Inhibition of Fru-1,6-P_2ase by α-PRibCP (•) at 5 μM Fru-1,6-P_2, α-PRibCP (▽) or β-PRibCP (○) at 20 μM Fru-1,6-P_2. Inset; Rib-1,5-P_2 at 5 μM (○) or 20 μM (•) Fru-1,6-P_2.

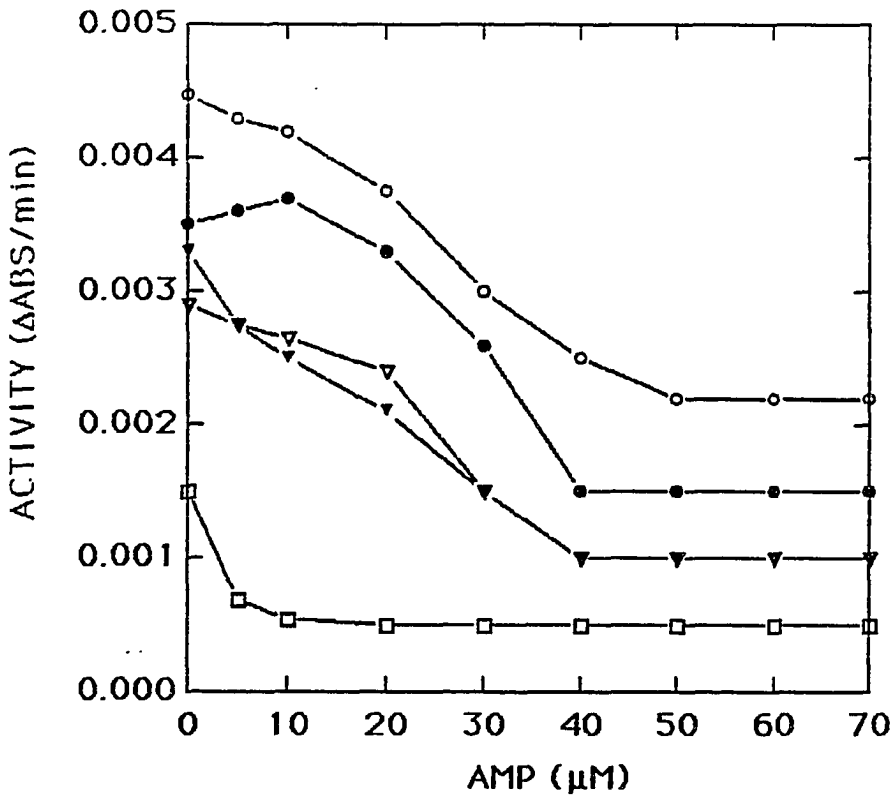


Figure 26: Potentiation of AMP inhbition of Fru-1,6-P_2ase. AMP alone (o) 60 μM Rib-1,5-P_2 (•), or α-PRibCP (▽), 2.0 μM (▼) or 20 μM (□) Fru-2,6-P_2. Fru-1,6-P_2 concentration was 5 μM

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