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Phosphorothioate Analogues of 5-Phosphoribosyl 1-Diphosphate: ^{31}P Nuclear Magnetic Resonance Study†

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ABSTRACT: ^{31}P nuclear magnetic resonance (NMR) has been used to study the 1-phosphorothioate analogues of 5-phosphoribosyl 1-diphosphate (P-Rib-PP). Comparison of the proton-decoupled spectra of 5-phosphoribosyl 1-*O*-(2-thiodiphosphate) (P-Rib-PPβS) and the S_P diastereomer of 5-phosphoribosyl 1-*O*-(1-thiodiphosphate) (P-Rib-PPαS) with the parent molecule revealed a characteristic large downfield chemical shift change for the resonance signal associated with the thiophosphate group ($\Delta\delta \sim 40$–50 ppm) and an increase in the magnitude of the phosphate–thiophosphate spin–spin coupling constant ($\Delta J_{\alpha\beta} \sim 10$ Hz). Both these changes are consistent with the observed effects of sulfur substitution on the behavior of the adenosine nucleotides, particularly ADP [Jaffe, E. K., & Cohn, M. (1978) *Biochemistry 17*, 652–657]. High-field ^{31}P NMR has also been used to demonstrate the diastereomeric purity of P-Rib-PPαS (S_P diastereomer) and the greater lability of this analogue when compared with both P-Rib-PPβS and P-Rib-PP. Sulfur substitution was found to cause a large decrease in the apparent pK_a associated with the thiophosphate moiety of P-Rib-PPβS (Δp$K_a \sim 1.4$ units) and also to enhance the sensitivity of the thiophosphate chemical shift to protonation and, in particular, to Mg^{2+} binding, compared with P-Rib-PP. The potential application of the phosphorothioate analogues as probes of the reactions catalyzed by the phosphoribosyltransferase enzymes is discussed.

The utility of ^{31}P NMR[1] in studies of phosphorus-containing molecules of biological importance and their interaction with diamagnetic metal ions, particularly Mg^{2+} (Tran-Dinh et al., 1975; Tran-Dinh & Neumann, 1977; Tran-Dinh & Roux, 1977; Nageswara Rao & Cohn, 1977; Bock, 1980; Bishop et al., 1981; Schliselfeld et al., 1982; Vogel & Bridger, 1982; Smithers & O'Sullivan, 1982), and also as a probe of enzyme-catalyzed reaction mechanisms and the structure and dynamics of enzyme–substrate complexes (Cohn & Nageswara Rao, 1979) has been well documented.

Although these applications of the technique appear to suffer from some limitations (Jaffe & Cohn, 1978a; Vogel & Bridger, 1982; Smithers & O'Sullivan, 1982), ^{31}P NMR has proved particularly rewarding in several studies involving phosphorothioate analogues. In a pioneering study, Jaffe & Cohn (1978a) elucidated differences in ^{31}P chemical shifts, spin–spin coupling constants, and apparent pK_a values between the phosphorothioate analogues of the adenosine nucleotides and their respective parent molecules. Of these differences, the change in pK_a upon sulfur substitution appears to provide a potential tool for probing the nature of metal–substrate–enzyme complexes where the pK_a of the bound metal–substrate differs significantly from that of the free complex (Jaffe & Cohn, 1978a; Cohn & Nageswara Rao, 1979). More recent applications include the direct determination of the equilibrium constants of phosphoryl group transfer reactions (Jaffe & Cohn, 1980; Lerman & Cohn, 1980) and studies of the coordination structure of free and enzyme-bound metal–ATP and metal–ADP complexes (Jaffe & Cohn, 1978b). ^{31}P NMR has also proved useful in distinguishing the diastereomers of several phosphorothioate analogues (Jaffe & Cohn, 1978a; Connolly et al., 1982; Orr et al., 1982). The technique thus provides a direct, nondestructive measure of diastereomeric purity and also the stereospecificity of enzymatic reactions involving these analogues [e.g., Orr et al. (1982)].

In the preceding paper (Smithers & O'Sullivan, 1984), we described the preparation and partial characterization of the 1-phosphorothioate analogues of P-Rib-PP. We present here the results of a ^{31}P NMR study of P-Rib-PPβS and the S_P diastereomer of P-Rib-PPαS. [Unfortunately, low yields in

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[1] Abbreviations: NMR, nuclear magnetic resonance; P-Rib-PP, 5-phosphoribosyl 1-diphosphate (the designations 5P and 1PP are used to denote the 5-phosphate and 1-diphosphate groups, respectively; $1P_\alpha$ indicates the phosphorus atom of the diphosphate group closest to the anomeric carbon of the ribose ring and $1P_\beta$ is the successively remote phosphorus atom); P-Rib-PPβS, 5-phosphoribosyl 1-*O*-(2-thiodiphosphate); P-Rib-PPαS, 5-phosphoribosyl 1-*O*-(1-thiodiphosphate); $J_{\alpha\beta}$, spin–spin coupling constant between the P_α and P_β nuclei; Rib-5-P, ribose 5-phosphate; SPP_i, thiopyrophosphate; δ, chemical shift (parts per million from 85% H_3PO_4); *R*, $[Mg]_T/[\text{phosphorylated ligand}]_T$; ADP$\beta$S, adenosine 5′-*O*-(2-thiodiphosphate); P_i, inorganic phosphate.

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the synthesis of the R_P diastereomer of P-Rib-PPαS prevented a similar study of this analogue (Smithers & O'Sullivan, 1984)].

Specifically, the effect of sulfur substitution on the chemical shifts and spin–spin coupling constant of P-Rib-PP is reported. In addition, the effects of pH and Mg^{2+} binding on the behavior of P-Rib-PPβS and the use of ^{31}P NMR to ascertain the stability and diastereomeric purity of the S_P diastereomer of P-Rib-PPαS are presented.

Experimental Procedures

Chemicals. The ammonium salts of P-Rib-PPβS and the S_P diastereomer of P-Rib-PPαS were synthesized and purified as described in the preceding paper (Smithers & O'Sullivan, 1984). Other chemicals were analytical reagent grade obtained from local suppliers or the sources described in Smithers & O'Sullivan (1982). Water was deionized and glass distilled.

Elimination of Paramagnetic and Other Trace Metal Ion Impurities. Procedures for the removal of metal ion impurities from glassware, distilled–deionized water, $MgCl_2$ solutions, acid (HCl) and alkali (KOH) for pH titrations, and P-Rib-PPβS and P-Rib-PPαS (S_P diastereomer) were as described previously (Smithers & O'Sullivan, 1982). The purified analogues were quantified by the orcinol procedure [cf. Smithers & O'Sullivan (1984)].

Standardization of $MgCl_2$. The metal ion solution was standardized by passing a measured volume of the solution through a Dowex cation-exchange resin (H^+ form) and titrating the effluent acid with standard hydroxide (O'Sullivan & Smithers, 1979).

^{31}P NMR Measurements. ^{31}P NMR spectra of P-Rib-PPβS were recorded at 40.3 MHz (^1H frequency = 99.6 MHz) on a JEOL FX-100 Fourier-transform NMR spectrometer equipped with quadrature phase detection. Spectrometer conditions used in the investigation were a sweep width of 5000 Hz (8192 data points) and a pulse width of 14 μs (flip angle ~90°). The pulse repetition rate varied from 2.5 to 5.0 s and the number of transients from 200 to 2000, depending upon the required signal to noise ratio of the resultant spectrum.

The ^{31}P NMR spectrum of P-Rib-PPαS (S_P diastereomer) was recorded at 121.5 MHz (^1H frequency = 300.1 MHz) on a Bruker CXP-300 Fourier-transform NMR spectrometer equipped with a tuneable multinuclear probe. Other spectrometer conditions included the following: sweep width, 15 000 Hz (8192 data points); pulse width, 10 μs (flip angle ~60°); acquisition time, 0.2731 s; number of transients, 4000. To increase the resolution of the spectrum following acquisition and prior to Fourier transformation, the free-induction decays were extended by zero filling to 16 384 data points. Spectra were recorded on samples (2.5 mL) contained within precision glass tubes (10 mm) and were broad-band proton noise decoupled.

^{31}P chemical shifts are reported with reference to 85% H_3PO_4, positive values being assigned to shifts downfield from the reference. The external H_3PO_4 reference and ^2H_2O (which served as the field frequency lock) were contained within a glass coaxial inner cell and, therefore, did not come in contact with the sample under analysis.

pH Titration. Titration of P-Rib-PPβS was carried out by the addition of small aliquots (1–5 μL) of HCl (1 or 2 M) to a solution (3 mL) of the analogue (3.6 mM) initially adjusted to pH ~8 with 1 M KOH. pH measurements were made with a Radiometer PHM 62 pH meter using a Radiometer semimicro combination glass electrode. The ^2H_2O, used as the field frequency lock, did not come in contact with the sample under investigation, and quoted pH values are actual pH readings.

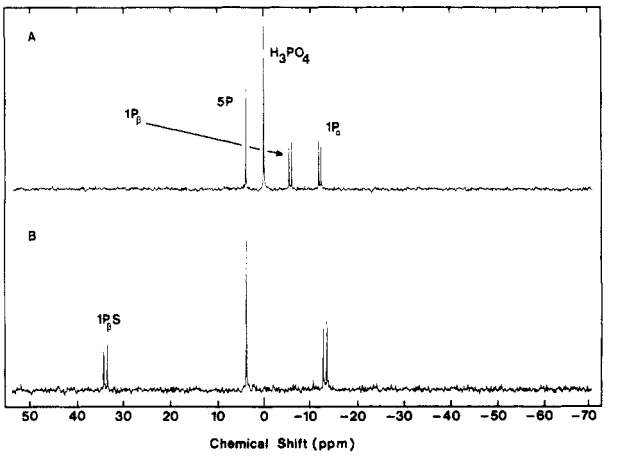

FIGURE 1: ^{31}P NMR spectra at 40.3 MHz (proton noise decoupled) of P-Rib-PP (5.4 mM) (A) and P-Rib-PPβS (3.8 mM) (B) at pH 8.6. Resonance signals were assigned by reference to the fully proton-coupled spectra of P-Rib-PP [cf. Smithers & O'Sullivan (1979)] and P-Rib-PPβS (not shown) and comparison of the proton-decoupled spectra of the two compounds. $T \sim 25$ °C.

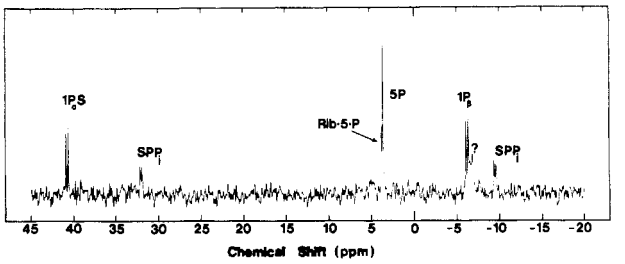

FIGURE 2: ^{31}P NMR spectrum at 121.5 MHz (proton noise decoupled) of the S_P diastereomer of P-Rib-PPαS (0.55 mM) at pH 8.45. Resonance signals associated with the molecule were assigned by reference to the proton-decoupled ^{31}P NMR spectrum of P-Rib-PP recorded under similar conditions. Peaks denoted by Rib-5-P and SPP_i represent breakdown products of P-Rib-PPαS, which appeared during accumulation of the spectrum. The peak designated by "?" represents an unknown contaminant present in the preparation. $T \sim 28$ °C.

Results

^{31}P NMR Spectra of the P-Rib-PP Analogues. A comparison of the proton noise-decoupled ^{31}P NMR spectra of P-Rib-PP and P-Rib-PPβS is presented in Figure 1, and the ^{31}P NMR spectrum of P-Rib-PPαS (S_P diastereomer) is depicted in Figure 2. Resonance signals were assigned by proton-coupled ^{31}P NMR spectrometry of P-Rib-PP [cf. Smithers & O'Sullivan (1979) and Smithers (1983)] and P-Rib-PPβS (not shown), and by comparison of the proton-decoupled spectra of P-Rib-PPβS (Figure 1) and P-Rib-PPαS (Figure 2) with the spectrum of the parent compound, on the basis of the observed chemical shifts, spin–spin multiplets, and coupling constant magnitudes. Relevant chemical shifts and coupling constants are compared with values for the phosphorothioate analogues of ADP and AMP in Table I.

The spectra depicted in Figures 1 and 2 were obtained with samples under alkaline conditions (pH >8), where P-Rib-PP, P-Rib-PPβS, and, presumably, P-Rib-PPαS would be present in their fully ionized (pentaanionic) forms (see Table II). Under these conditions, sulfur substitution for a nonbridging oxygen atom at the diphosphate moiety resulted in large downfield shifts in the thiophosphate resonances, ~40 and ~50 ppm for substitution at the β- and α-positions, respectively, compared to P-Rib-PP. Resonance signals arising from the unsubstituted phosphorus groups were only slightly affected. The phosphate–thiophosphate coupling constants of both analogues ($J_{\alpha\beta} \sim 30$ Hz) were characteristic of –P–O–

Table I: Changes in the ^{31}P Chemical Shifts and Spin–Spin Coupling Constant of P-Rib-PP upon Sulfur Substitution of a Nonbridging Oxygen Atom at the Diphosphate Group: Comparison with the Phosphorothioate Analogues of ADP and AMP[a]

phosphorylated compd	pH[b]	charge on thiophosphate group	$\Delta\delta_{P(S)}$ (ppm)[c]	$\Delta J_{\alpha\beta}$ (Hz)[d]
P-Rib-PPβS	8.20	2−	39.6	10.2
	2.90	1−	49.5	10.7
P-Rib-PPαS (S_P diastereomer)	8.45	1−	52.85	9.0
ADPβS	~8	2−	39.2	9.5
ADPαS	~8	1−	51.6	8.6
AMPS	~8	2−	39.4	

[a] Data for the phosphorothioate analogues of ADP and AMP were taken from Jaffe & Cohn (1978a). [b] Uncertainty in the pH values is 0.01 pH unit. [c] Downfield chemical shift change of the thiophosphate group, relative to the shift of the parent compound. Uncertainty in the chemical shifts is 0.03 (P-Rib-PPβS) and 0.09 (P-Rib-PPαS) ppm. [d] Increase in the ^{31}P spin–spin coupling constant, relative to the coupling constant of the parent compound. Uncertainty in the coupling constants is 1.2 (P-Rib-PPβS) and 3.7 (P-Rib-PPαS) Hz.

P(S)− groups (Jaffe & Cohn, 1978a) and were ~10 Hz greater than the phosphate–phosphate coupling constant recorded for P-Rib-PP (Table I).

Stability and Diastereomeric Purity of P-Rib-PPαS (S_P Diastereomer). The introduction of a sulfur atom at the $1P_\alpha$ moiety of P-Rib-PP increased the lability of the molecule. Resonance signals associated with Rib-5-P and SPP_i, products resulting from the hydrolysis of P-Rib-PPαS [cf. Smithers & O'Sullivan (1979)], were evident in the ^{31}P NMR spectrum of this analogue (Figure 2). On the basis of the time-dependent increase in the peak height of the Rib-5-P signal, compared with the 5P resonance, the estimated half-life of P-Rib-PPαS at 28 °C was approximately 4 h. The increased lability of this analogue, compared with both P-Rib-PP and P-Rib-PPβS, and the limited quantities available (Smithers & O'Sullivan, 1984) prevented a more detailed ^{31}P NMR study.

Diastereomeric pairs of phosphorothioate analogues can be distinguished by ^{31}P NMR, on the basis of differences between the pairs in the chemical shift of the thiophosphate resonance (Sheu & Frey, 1977; Jaffe & Cohn, 1978a; Orr et al., 1982). These differences have been attributed to a slight variation in geometric configuration between the R_P and S_P forms, since ^{31}P chemical shifts are particularly sensitive to alterations in molecular geometry (Gorenstein et al., 1976). Thus, in the spectral region associated with the $1P_\alpha$ doublet (δ = 40.7 ppm) of the S_P diasteromer of P-Rib-PPαS (Figure 2), and on the basis of the limitations imposed by the resonance signal amplitudes and noise level of the spectrum, other resonances (arising from possible contamination with the R_P diastereomer) could not be observed. This was as expected, on the basis of the strict stereospecificity of the P-Rib-PP synthetase reaction and resolution of the two isomers by DEAE-Sephadex chromatography (Smithers & O'Sullivan, 1984).

Effect of $MgCl_2$ on the ^{31}P Chemical Shifts of P-Rib-PPβS. Proton noise-decoupled ^{31}P NMR spectra of P-Rib-PPβS at pH 8.2, in the absence and presence of an equimolar concentration of $MgCl_2$, are compared in Figure 3. At this pH, where P-Rib-PPβS is fully ionized (P-Rib-PPβS^{5-}) (see Table II), the addition of $MgCl_2$ resulted in a large downfield shift in the $1P_\beta$ resonance ($\Delta\delta$ = 1.39 ppm) and a smaller shift in the adjacent $1P_\alpha$ resonance ($\Delta\delta$ = 0.26 ppm) (Figure 3B) when compared with the spectrum in the absence of metal ions (Figure 3A). No shift in the 5P resonance could be detected. In contrast, under conditions where P-Rib-PP is also fully

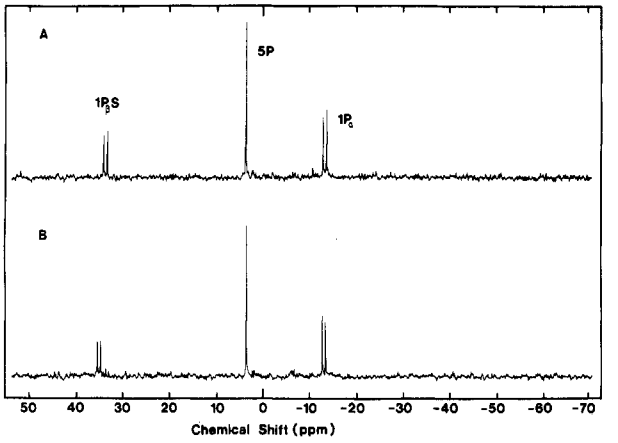

FIGURE 3: ^{31}P NMR spectra at 40.3 MHz (proton noise decoupled) of P-Rib-PPβS (3.6 mM) in the absence (A) and presence (B) of an equimolar concentration of $MgCl_2$ at pH 8.2. $T \sim 25$ °C.

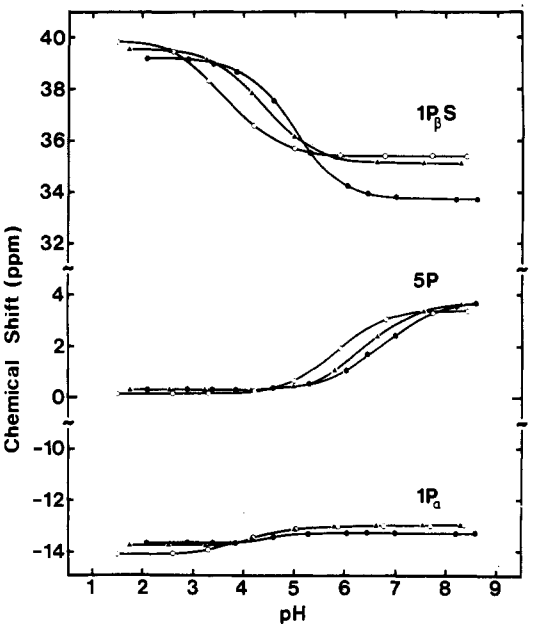

FIGURE 4: ^{31}P NMR pH titration curves of P-Rib-PPβS (3.6 mM) in the absence and presence of various concentrations of $MgCl_2$: $R = [Mg]_T/[\text{P-Rib-PP}\beta\text{S}]_T = 0$ (●); $R = 1$ (▲); $R = 10$ (○). Chemical shifts are reported in parts per million from 85% H_3PO_4, positive values being assigned to shifts downfield from the reference. $T \sim 25$ °C.

ionized, the addition of an equimolar concentration of $MgCl_2$ resulted in a much smaller change in the shift of the $1P_\beta$ resonance (Smithers & O'Sullivan, 1982).

pH Titration of P-Rib-PPβS. The effect of $MgCl_2$ on the ^{31}P chemical shifts of P-Rib-PPβS (Figure 3) was further investigated by carrying out ^{31}P NMR pH titrations of the analogue over the range pH 1–9, in the absence and presence of $MgCl_2$. Chemical shifts and spin–spin coupling constants were determined as a function of pH with P-Rib-PPβS alone at 3.6 mM and in the presence of two concentrations of added $MgCl_2$, 3.6 and 36 mM, respectively. These concentrations correspond to values for R, the ratio of added $MgCl_2$ to total P-Rib-PPβS ($R = [Mg]_T/[\text{P-Rib-PP}\beta\text{S}]_T$), of 1.0 and 10.0, respectively. The titration curves obtained are shown in Figure 4.

A comparison of the variation in the chemical shifts of the $1P_\beta$ resonance signals of P-Rib-PPβS (taken from Figure 4) and P-Rib-PP [taken from Smithers & O'Sullivan (1982)] as a function of pH, in the absence of $MgCl_2$ (R = 0), is depicted in Figure 5. Sulfur substitution had two effects on the behavior of the $1P_\beta$ resonance during pH titration; viz., the

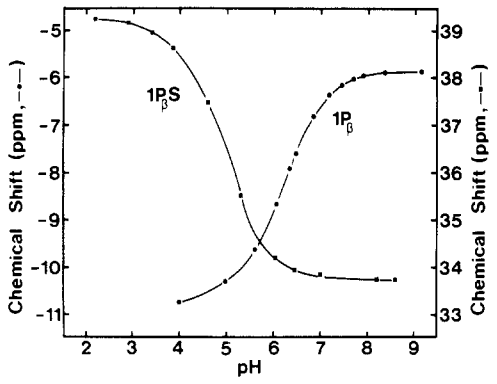

FIGURE 5: Comparison of the variation in the chemical shifts of the $1P_\beta$ resonance signals of P-Rib-PP [8.3 mM (●)] and P-Rib-PPβS [3.6 mM (■)] as a function of pH. Data for P-Rib-PP and P-Rib-PPβS were taken from Smithers & O'Sullivan (1982) and Figure 4, respectively. Chemical shifts are reported in parts per million from 85% H_3PO_4, positive values being assigned to shifts downfield from the reference. $T \sim 25$ °C.

direction of the chemical shift change was reversed, and the magnitude of the chemical shift change was increased. Protonation of the dianionic $1P_\beta$ moiety of P-Rib-PPβS resulted in a large downfield shift ($\Delta\delta \sim 5.5$ ppm) in this resonance, whereas titration of the $1P_\beta$ group of the parent molecule resulted in an upfield shift ($\Delta\delta \sim 5$ ppm) in this resonance (Figure 5). Similar behavior has been reported for the phosphorothioate analogues of ATP, ADP, AMP, and P_i when compared with their respective parent molecules (Jaffe & Cohn, 1978a).

The points of inflexion (apparent pK_a values) of the titration curves shown in Figure 4 were calculated from their second derivatives by using a BASIC curve-analysis computer program that incorporated the rationale and smoothing algorithm reported by Reinsch (1967). These calculations were carried out by Dr. H. O. Spivey and his colleagues at the Oklahoma State University, Stillwater, OK. Results are presented in Table II and values for P-Rib-PP [calculated from data reported by Smithers & O'Sullivan (1982)] and ADP and ADPβS (Jaffe & Cohn, 1978a) have been included for comparison.

In the absence of $MgCl_2$ ($R = 0$), titration of P-Rib-PPβS over the pH range 1–9 demonstrated that the magnitude of the chemical shift variations was in the order of $1P_\beta > 5P \gg 1P_\alpha$ (Figure 4), consonant with the behavior of P-Rib-PP over a similar pH range (Smithers & O'Sullivan, 1982). However, the apparent pK_a associated with the $1P_\beta$ protonation was sensitive to sulfur substitution, with a value of 1.4 units below the corresponding pK_a of P-Rib-PP, in substantial agreement with the pK_a variation reported for the P_β oxyanion of ADPβS ($\Delta pK_a = -1.6$ units) (Table II). Sulfur substitution also had a second-order effect on the apparent pK_a associated with the $1P_\alpha$ oxyanion. Although the chemical shift variation of this resonance during titration was of the same direction and magnitude as observed for P-Rib-PP, the apparent pK_a was reduced by 2.0 units, consistent with the lower pK_a of the $1P_\beta$ moiety (Table II). In contrast, sulfur substitution had little effect on the apparent pK_a associated with protonation of the 5P moiety ($\Delta pK_a = -0.15$ unit).

The presence of an equimolar concentration of $MgCl_2$ ($R = 1$) reduced the apparent pK_a associated with the $1P_\beta$ moiety, while the pK_a of the 5P group was not significantly altered (Figure 4, Table II). The presence of a large excess of $MgCl_2$ ($R = 10$) reduced the apparent pK_a values associated with the $1P_\beta$ oxyanion ($\Delta pK_a \sim 1.2$ units) and the 5P moiety ($\Delta pK_a \sim 0.6$ unit) (Table II).

Table II: Apparent pK_a Values Associated with the Phosphooxyanion Moieties of P-Rib-PPβS in the Absence and Presence of $MgCl_2$: Comparison with P-Rib-PP, ADP, and ADPβS[a]

phosphorylated compd[b]	R[c]	apparent pK_a[d] 5P	P_α	P_β
P-Rib-PPβS	0	6.27 (−0.15)	4.29 (−2.0)	4.96 (−1.4)
	1	6.32	4.47	4.45
	10	5.72	3.68	3.72
P-Rib-PP	0	6.42	6.29	6.36
	1	6.38	5.23	5.25
	10	5.58	4.32	4.37
ADPβS	0			5.2 (−1.6)
ADP	0			6.8

[a] The pK_a values for P-Rib-PPβS and P-Rib-PP were determined from the second derivatives of the titration curves depicted in Figure 4 and the data reported by Smithers & O'Sullivan (1982), respectively, by using a BASIC curve-analysis computer program. These calculations were carried out by Dr. H. O. Spivey and his colleagues at the Oklahoma State University, Stillwater, OK. The pK_a values for ADP and ADPβS were taken from Jaffe & Cohn (1978a). [b] For P-Rib-PPβS and P-Rib-PP, P_α indicates the phosphorus atom closest to the anomeric carbon of the ribose ring. For ADPβS and ADP, P_α indicates the phosphorus atom closest to adenosine. [c] $R = [Mg]_T/[\text{phosphorylated compound}]_T$. [d] Values in parentheses refer to the change in pK_a upon sulfur substitution. Values at $R = 1$ do not represent a single dissociation as the extent of complexation with Mg^{2+} changes during the titration (H. O. Spivey, personal communication).

Table III: Comparison of the ^{31}P Chemical Shifts and Spin–Spin Coupling Constants of P-Rib-PPβS, P-Rib-PPαS (S_P Diastereomer), ADPβS, and P-Rib-PP in the Absence and Presence of $MgCl_2$ at Basic and Acidic pH Values

phosphorylated compd[a]	pH[b]	R[c]	δ (ppm)[d] 5P	P_α	P_β	$J_{\alpha\beta}$ (Hz)
P-Rib-PPβS[e]	2.90	0	0.31	−13.66	39.16	31.2
	8.20	0	3.56	−13.28	33.73	32.4
	2.52	1	0.28	−13.81	39.43	28.1
	8.28	1	3.56	−13.02	35.12	28.0
	2.62	10	0.19	−14.10	39.40	27.5
	7.72	10	3.34	−13.08	35.42	27.5
P-Rib-PPαS[f]	8.45	0	3.55	40.73	−6.27	31.2
ADPβS[g]	8.0	0		−11.1	33.9	31.2
	8.0	1		−11.0	35.2	28.1
P-Rib-PP[h]	4.94	0	0.27	−12.89	−10.31	20.5
	9.14	0	3.66	−12.12	−5.87	22.2
	4.66	1	0.24	−12.74	−9.68	19.8
	9.00	1	3.61	−11.84	−5.81	19.3
	3.99	10	0.05	−12.97	−9.56	16.5
	8.09	10	3.11	−11.95	−6.29	16.0

[a] For P-Rib-PP and its derivatives, P_α indicates the phosphorus atom closest to the anomeric carbon of the ribose ring. For ADPβS, P_α indicates the phosphorus atom closest to adenosine. [b] Uncertainty in the pH values is 0.01 pH unit. [c] $R = [Mg]_T/[\text{phosphorylated compound}]_T$. [d] Relative to 85% H_3PO_4 external reference. Positive values are downfield. [e] Uncertainty in the chemical shifts is 0.03 ppm and in the coupling constants is 1.2 Hz. [f] Uncertainty in the chemical shifts is 0.09 ppm and in the coupling constant is 3.7 Hz. [g] Data taken from Jaffe & Cohn (1978a). [h] Data taken from Smithers & O'Sullivan (1982).

Selected ^{31}P chemical shifts and coupling constants from the titration of P-Rib-PPβS (Figure 4) are compared with results from similar experiments carried out with P-Rib-PP (Smithers & O'Sullivan, 1982) and ADPβS [taken from Jaffe & Cohn (1978a)] and also with results from the ^{31}P NMR spectrum of P-Rib-PPαS (Figure 2) in Table III.

Sulfur substitution at the diphosphate moiety of P-Rib-PP not only resulted in a large downfield shift in the thiophosphate resonance but also induced a concomitant small upfield shift in the resonance arising from the adjacent phosphooxyanion, when compared with the parent molecule. Under fully ionizing conditions (pH ~8), the chemical shift of the $1P_\beta$ resonance of P-Rib-PPβS was almost equivalent to that reported for the

Table IV: Changes in the ^{31}P Chemical Shifts of the Diphosphate Resonances of P-Rib-PPβS, ADPβS, and P-Rib-PP in the Presence of Equimolar $MgCl_2$[a]

phosphorylated compd[b]	Δδ (ppm)[c]	
	P_α	P_β
P-Rib-PPβS	0.26	1.39
ADPβS	0.10	1.30
P-Rib-PP	0.28	0.06

[a] The values were calculated from results under fully ionizing conditions (pH ~8) (Table III). [b] For P-Rib-PPβS and P-Rib-PP, P_α indicates the phosphorus atom closest to the anomeric carbon of the ribose ring. For ADPβS, P_α indicates the phosphorus atom closest to adenosine. [c] Downfield chemical shift change, relative to the shift in the absence of metal ion.

P_β resonance of ADPβS, whereas the $1P_\alpha$ resonance was shifted upfield approximately 2.5 ppm when compared with the shift of the P_α resonance of ADPβS. This phenomenon may be related to differences between the C_1 atom (adjacent to $1P_\alpha$ in P-Rib-PPβS) and the C_5 atom (adjacent to P_α in ADPβS), as a comparison of the respective parent molecules also revealed a similar difference in the chemical shifts of the P_α signals (Smithers & O'Sullivan, 1982). On the other hand, sulfur substitution at the diphosphate moiety had little effect on the behavior of the distant 5P group. The spin–spin coupling constant of both P-Rib-PP analogues ($J_{\alpha\beta} \sim 30$ Hz) was equivalent to that reported for ADPβS and, in the case of P-Rib-PPβS, was relatively independent of pH variation ($\Delta J_{\alpha\beta} = 1.2$ Hz) (Table III).

In the presence of an equimolar concentration of $MgCl_2$ ($R = 1$), the $1P_\alpha$ and $1P_\beta$ resonances of P-Rib-PPβS were shifted in the expected downfield direction [Table IV; cf. Smithers & O'Sullivan (1982)]. The $1P_\beta$ shift ($\Delta\delta = 1.39$ ppm) was very similar to the P_β shift reported for ADPβS ($\Delta\delta = 1.30$ ppm) but was enhanced some 20-fold when compared with the $1P_\beta$ shift of the parent molecule ($\Delta\delta = 0.06$ ppm). The $1P_\alpha$ shift ($\Delta\delta = 0.26$ ppm) paralleled that for P-Rib-PP. The reduction in the spin–spin coupling constant of P-Rib-PPβS ($\Delta J_{\alpha\beta} = 4.4$ Hz), under fully ionizing conditions (pH >8), was in substantial agreement with that reported for ADPβS ($\Delta J_{\alpha\beta} = 3.1$ Hz) and remained insensitive to protonation over the pH range investigated. There was a negligible shift for the 5P resonance of P-Rib-PPβS (Table III).

Discussion

The ^{31}P NMR behavior of the phosphorothioate analogues of P-Rib-PP closely parallels that reported for the phosphorothioate analogues of the adenosine nucleotides, particularly ADPβS (Jaffe & Cohn, 1978a), and reflects the similarity of ribose–phosphate components of the respective parent molecules (Thompson et al., 1978; Smithers & O'Sullivan, 1982). The large downfield shifts measured for the thiophosphate resonances of P-Rib-PPβS (Figure 1) and the S_P diastereomer of P-Rib-PPαS (Figure 2) and the magnitude of the phosphate–thiophosphate coupling constants are characteristic of phosphorothioate esters, in which a nonbridging oxygen atom is replaced with sulfur. Changes observed in these parameters closely paralleled those reported for the phosphorothioate analogues of ADP and AMP (Jaffe & Cohn, 1978a) (Table I). The small changes observed in the chemical shifts of ^{31}P resonances arising from the unsubstituted oxyanions, when compared with P-Rib-PP, suggest that major differences in the orientation of the phosphate groups of these molecules in solution do not occur. Therefore, the large chemical shift change of the thiophosphate resonances most probably results from the difference in electronic structure between sulfur and oxygen and, to a much lesser extent, a variation in bond angle, also known to cause changes in ^{31}P chemical shifts (Gorenstein et al., 1976).

The substantial reduction in the apparent pK_a associated with the thiophosphate moiety of P-Rib-PPβS, when compared with the phosphate moiety of the parent molecule (Table II), is in excellent agreement with that reported for ADPβS (Jaffe & Cohn, 1978a) and is characteristic of phosphorothioate analogues in general. This pK_a variation, accompanying sulfur substitution, offers an explanation for the late elution of the P-Rib-PP analogues from DEAE-Sephadex, noted during their purification (Smithers & O'Sullivan, 1984).

The observed downfield changes in the $1P_\alpha$ and $1P_\beta$ resonances of P-Rib-PPβS (Figure 3, Table IV), the reduction in the phosphate–thiophosphate coupling constant (Table III), and variation in the pK_a of the $1P_\beta$ oxyanion (Figure 4, Table II) upon chelation with Mg^{2+} ions are similar to the changes reported in these parameters for the $MgADP\beta S^-$ complex (Jaffe & Cohn, 1978a). Although sulfur substitution increases the sensitivity of the thiophosphate chemical shift to Mg^{2+} binding (Table IV), these changes also follow an effectively identical trend with the ^{31}P NMR behavior of the magnesium complexes of P-Rib-PP and ADP (Smithers & O'Sullivan, 1982). It would appear that, in solution, P-Rib-PPβS and ADPβS display a similar mode of metal ion binding, analogous to the respective parent molecules (Thompson et al., 1978; Smithers & O'Sullivan, 1982).

Two potentially rewarding applications, particularly for P-Rib-PPβS, lie in studies of the nature of the metal–P-Rib-PP–phosphoribosyltransferase complex. First, the reduced pK_a of P-Rib-PPβS, particularly in the presence of $MgCl_2$ (Table II), allows the possibility of more accurate kinetic and magnetic resonance analyses of the phosphororibosyltransferase enzymes with acidic pH optima, exemplified by quinolinate phosphoribosyltransferase. At the pH optimum of the hog kidney enzyme (pH 5.5) (Shibata & Iwai, 1980), P-Rib-PPβS is essentially fully ionized in the presence of $MgCl_2$ (Table II) so that only three species ($P\text{-Rib-PP}\beta S^{5-}$, $P\text{-Rib-PP}\beta SMg^{3-}$, and $MgP\text{-Rib-PP}\beta SMg^-$) would reach a significant concentration. At this pH, the parent molecule is only partially ionized, and the addition of Mg^{2+} produces a situation of considerable complexity with respect to the variety of species present (O'Sullivan & Smithers, 1979). Second, the binding of $MgADP^-$ to the active site of kinase enzymes can result in a significant change in the pK_a of the P_β moiety (Nageswara Rao & Cohn, 1977). As similar changes could occur for the Mg^{2+} complex of P-Rib-PP when enzyme bound and since sulfur substitution reduces the pK_a of the $1P_\beta$ oxyanion of P-Rib-PPβS (Table II), this analogue could be exploited in probing the nature of the enzyme active site, in a manner similar to that proposed for ADPβS (Jaffe & Cohn, 1978a).

The increased lability of P-Rib-PPαS (S_P diastereomer) with the formation of Rib-5-P and SPP_i, noted during accumulation of the ^{31}P NMR spectrum of the analogue (Figure 2), can be explained, in part, by thermodynamic considerations. The equilibrium of reactions involving phosphorothioate analogues favors the formation of a molecule containing a terminal thiophosphate group from one containing an internal thiophosphate group (Jaffe & Cohn, 1980). Such a shift in equilibrium, originally observed for the reaction catalyzed by aminoacyl-tRNA synthetase, in which ATPβS is converted to ATPγS (Rossomando et al., 1979), has since been reported for the reactions catalyzed by phosphoglycerate kinase (Jaffe & Cohn, 1980) and creatine and arginine kinase (Lerman & Cohn, 1980), in which ATPβS is converted to ADPβS. It was

possible to use ^{31}P NMR to make a direct determination of the equilibrium constants of these reactions. This was demonstrated, in particular, for the phosphoglycerate kinase reaction, which is essentially irreversible in the presence of the natural substrate (Jaffe & Cohn, 1980). Since the equilibrium of the phosphoribosyltransferase reactions usually favors the formation of PP_i from P-Rib-PP (Srivastava & Beutler, 1971; Giacomello & Salerno, 1978), use of the phosphorothioate analogues of P-Rib-PP in a similar application may also be rewarding.

In conclusion, the availability of the P-Rib-PP analogues and the ^{31}P NMR study reported here provide the basis for a study of the phosphoribosyltransferase enzymes, analogous to the approach pioneered by Cohn and her co-workers with the kinase enzymes (Cohn, 1982).

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