The Synthesis and Metal Binding Characteristics of Novel, Isopolar Phosphonate Analogues of Nucleotides

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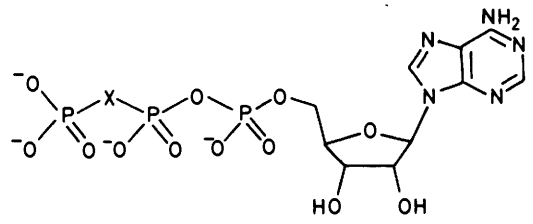
Dichloromethylene-, difluoromethylene-, and fluoromethylene-bisphosphonic acids condense with the 5'-phosphoromorpholidates of adenosine and guanosine to give nucleotide analogues of ATP and GTP which are isopolar and isosteric. An isopolar but non-isosteric analogue of ATP results from the condensation of acetylene-1,2-bisphosphonic acid with AMP-morpholidate.

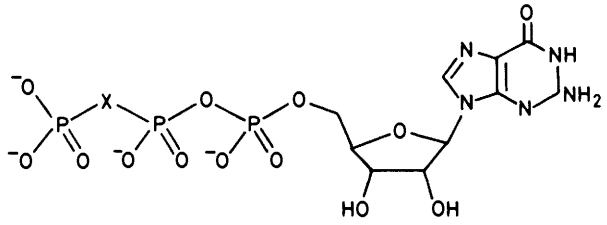
^{31}P N.m.r. and pK_a data show that the physical analogy improves in the series $CH_2 < CCl_2 \leqslant CFH < CF_2 < C{\equiv}C < O$ with respect to the β,γ-bridging function. N.m.r. and ion-selective electrode measurements on the binding of the divalent metals Mg, Ca, Zn, and Ba suggest that the pattern of metal cation binding to the nucleotide tetra-anion responds both to steric and to electronic features of the phosphonates, with the β,γ-difluoromethylene analogue most closely resembling ATP.

Phosphonate derivatives of nucleotides, in which the oxygen of a P–O–P or P–O–C link is replaced by a methylene group, have much potential as analogues of many biologically active phosphates. Myers [1] prepared the first analogue of adenosine 5'-triphosphate, ATP(**1a**) by condensing methylenebisphosphonic acid with adenosine 5'-phosphoromorpholidate to produce 5'-adenylylmethylenebisphosphonic acid (**1h**). Similar analogues of ATP, ADP, GTP (**2a**), and GDP having α,β- or β,γ-methylene groups have been prepared.[2]



(**1**)

a; X = O
b; X = NH
c; X = CF_2
d; X = CFH
e; X = CCl_2
f; X = C≡C
g; X = O—O
h; X = CH_2



(**2**)

a; X = O
b; X = CF_2
c; X = CFH
d; X = CCl_2

Their description has been abbreviated *e.g.* for (**1h**) to β,γ-methylene adenosine triphosphate and further shortened to $AMPPCH_2P$.

These compounds are sensibly isosteric with the parent nucleotide and enjoy the benefit of stable P–C bonds replacing scissile P–O–C linkages. However, they have experienced but limited success relative to phosphorimidate [3] and phosphorothioate [4] analogues when used in enzyme studies. One general analysis of the problem has led to the conclusion that shape is a criterion of possibly lesser significance in the design of phosphonate analogues than are the features relating to its electron distribution.[5] To test this proposition we have made several nucleotide analogues of ATP and GTP covering the range from the isosteric but non-isopolar methylenebisphosphonates, through the isosteric and isopolar halogenomethylenebisphosphonates, to the isopolar but non-isosteric acetylene-1,2-bisphosphonate.

Under physiological conditions, ADP and ATP exist predominantly bound to magnesium in 1:1 complexes. Moreover, metal cation binding to phosphates is a key feature in the enzymic catalysis of many phosphoryl transfer processes.[6] Accordingly we report some physical properties of the nucleotide analogues as their salts with mono- and di-valent metal cations with especial reference to ^{31}P n.m.r. behaviour. Part of this work has appeared in a preliminary report.[7]

Experimental

Materials.—Adenosine 5'-phosphoromorpholidate and guanosine 5'-phosphoromorpholidate were purchased from Sigma London Chemical Co. Ltd. Bisphosphonates were prepared as their tetra-alkyl esters and converted into the free acids by means of iodotrimethylsilane [8] and immediately neutralised with freshly distilled tri-n-butylamine to form the corresponding bis-tri-n-butylammonium salts. All other reagents, buffers, and salts were of AnalaR grade. DEAE-Sephadex was from Pharmacia. Glass-distilled water was used throughout.

Melting points were determined with a Kofler hot-stage micromelting point apparatus and are otherwise uncorrected. Determinations of pK_a were performed using a titration method with a Radiometer ABU12, TTT11, PHM28, and SBR2c apparatus with a GK2321c combination electrode. Calcium and barium ion-selective electrodes were obtained from EDT Research (London) and used in conjunction with a Solartron 7051 digital microvoltmeter. Divalent metal ion activities were determined for carbonate-free solutions of nucleotide analogues (0.5 to 1.2 mM) containing 1.00 equivalents of MCl_2 at ionic strength 0.1 (KNO_3) with pH adjusted to 8—8.5. H.p.l.c. was carried out on a Waters 440 system using a 25 cm column packed with Machery and Nagel Nucleosil 10SB and with pH 4.5 buffer (200 mM phosphate, 300 mM acetate) as eluant at 4 000 lb in^{-2}, 4 ml min^{-1}.

N.m.r. Analyses.—Routine spectra were recorded for ^{31}P in 10% D_2O solutions at 40.48 MHz with a Jeol JNM-PS-100 and at 162 MHz with a Bruker WH 400 machine, using 85% phosphoric acid as external reference, and with broad-band proton decoupling except as specified. For pH titrations, nucleotide analogues were dissolved in 30% D_2O solution at 20 mM concentration with 100 μM EDTA and spectra were recorded at 81 MHz on a Bruker WM 200 instrument. A typical experiment used 3.4 s acquisition time, 2 403 Hz sweep width, and 640 scans. Such spectra were sufficiently accurate for determination of both chemical shift and coupling constant data. Upfield shifts take negative sign by the usual convention.

pH Measurements of samples (1.5 ml) in 10 mm precision tubes were made using a Russell CTWL combination electrode inserted into the sample in the n.m.r. tube and pH adjustments were made by adding aliquots of 2M-HCl from a micropipette and vortexing the sample. No corrections for solvent deuteriation have been used. Magnesium complexes were prepared by addition of 1.1 equivalents of $MgCl_2$ at low pH and the pH raised by successive addition of aliquots of 2M-NaOH from a micropipette. Calcium, magnesium, and zinc complexes were prepared by addition of 1.1 equivalents of MCl_2 to samples at pH 9.1.

^{19}F N.m.r spectra were recorded at 94.08 MHz on the Jeol machine relative to external $CFCl_3$ reference. ^1H N.m.r. spectra were recorded on a Perkin-Elmer R34 spectrometer at 220 MHz or on the Bruker WH 400 at 400 MHz using $SiMe_4$ as external reference.

Nucleotide purities were checked by h.p.l.c. prior to n.m.r.–pH titrations and found to be more than 99% pure.

Adenosine 5'-(β,γ-μ-Difluoromethylene)triphosphate, AMPPCF$_2$P (**1c**).—(*a*) Morpholine-4-*N*,*N*'-dicyclohexylcarboxamidinium adenosine-5'-phosphoromorpholidate[9] (390 mg, 0.5 mmol) was dissolved in anhydrous, amine-free pyridine (5 ml). The solution was evaporated to dryness and the procedure repeated twice more with exclusion of moisture. It was finally dissolved in pyridine (3 ml). Similarly the bis(tri-n-butylammonium) salt of difluoromethylenebisphosphonic acid[7] (580 mg, 1.0 mmol) was evaporated repeatedly from its solution in pyridine (3 × 5 ml). Finally, the two pyridine solutions were combined and evaporated to dryness. The residue was kept in anhydrous pyridine (4 ml) for 24 h with magnetic stirring and exclusion of moisture. After this time the solution was evaporated to remove pyridine. The residue was dissolved in deionised water (5 ml), applied to a column of DEAE-Sephadex (3 × 30 cm), and the product eluted with a linear salt gradient (0—0.5M-LiCl). Fractions containing the analogue were combined and evaporated to dryness. The white solid residue was dissolved in a small volume of anhydrous methanol (5 ml) and the nucleotide precipitated out by the addition of acetone (25 ml). The precipitated product was collected by centrifugation and the whole procedure repeated four times. The white pellet was finally redissolved in methanol (10 ml) and evaporated to dryness to yield the white, powdery *product* as the tetralithium salt (164 mg, 54%), m.p. 225—235 °C (decomp.) (Found: C, 21.55; H, 2.1; N, 12.05. $C_{11}H_{12}F_2Li_4N_5O_{12}P_3 \cdot 2H_2O$ requires C, 21.96; H, 2.66; N, 11.64%); $\delta_P(D_2O$ pH 7.5) −10.4 (d, P_α, $^2J_{\alpha\beta}$ 32 Hz), −2.2 (ddt, P_β, $^2J_{\alpha\beta}$ 32 Hz, $^2J_{P\beta F}$ 89.5 Hz, $^2J_{\beta\gamma}$ 58.0 Hz), +4.25 (dt, P_γ, $^2J_{\beta\gamma}$ 58.0 Hz, $^2J_{P\gamma F}$ 70.2 Hz); $\delta_F(D_2O)$ −118.61 (dd, $^2H_{FP\gamma}$ 89.5 Hz, $^2J_{FP}$ 70.2 Hz); pK_a[4] = 6.7 ± 0.1 (at 3 mM).

(*b*) An alternative purification using DEAE cellulose chromatography with triethylammonium hydrogen carbonate buffer (0.05—0.2 M, pH 7.6) followed by chromatography on Bio-Gel P2 gave the *product* as its bis(triethylammonium) salt (11.1%), m.p. 220—230 °C (decomp.) (Found: C, 32.1; H, 6.3; N, 10.95. $C_{11}H_{16}F_2N_5O_{12}P_3 \cdot 2C_6H_{15}N \cdot 7H_2O$ requires C, 31.76; H, 6.90; N, 11.27%).

Adenosine 5'-(β,γ-μ-Monofluoromethylene)triphosphate, AMPPCFHP (**1d**).—In a reaction analogous to that for (**1c**) above, the title compound was prepared from the reaction of morpholine-4-*N*,*N*'-dicyclohexylcarboxamidinium adenosine-5'-phosphoromorpholidate (144 mg, 0.2 mmol) and the bis(tri-n-butylammonium) salt of monofluoromethylenebisphosphonic acid[7] (564 mg, 1 mmol) in anhydrous pyridine. The *product* was obtained as a colourless glass (29 mg, 17.4%), m.p. 210—220 °C (decomp.) (Found: C, 33.7; H, 6.95. $C_{11}H_{17}FN_5O_{12}P_3 \cdot 2C_6H_5N \cdot 6H_2O$ requires C, 33.13; H, 7.08%); $\delta_P(D_2O$ pH 7.0) −10.48 (d, P_α, $^2J_{\alpha\beta}$ 29.0 Hz), +2.42 (dm, P_β, $^2J_{P\beta F}$ 57 Hz), +8.58 (dm, P_γ, $^2J_{P\gamma F}$ 60 Hz); $\delta_F(D_2O)$ −218.25 (dd, $^2J_{FP\beta}$ 57 Hz, $^2J_{FP\gamma}$ 60 Hz); pK_a[4] 7.45 ±0.1 (at 3 mM).

Adenosine 5'-(β,γ-μ-Dichloromethylene)triphosphate, AMPPCCl$_2$P (**1e**).—In a reaction analogous to preparation (*a*) of (**1c**) morpholine-4-*N*,*N*'-dicyclohexylcarboxamidinium adenosine-5'-phosphoromorpholidate (390 mg, 0.5 mmol) was condensed with the bis(tri-n-butylammonium) salt of dichloromethylenebisphosphonic acid[10] (613 mg, 1 mmol). The product was chromatographed on DEAE Sephadex using a linear salt gradient (LiCl, 0—0.5 M, pH 7.0, 2 l). Fractions containing the product were combined and evaporated to dryness and the *product* isolated by repeated dissolution in methanol (5 ml) and precipitation with acetone (25 ml) to give a white powder (247 mg, 75.7%), m.p. 235—245 °C (decomp.) (Found: C, 19.7; H, 2.65. $C_{11}H_{12}Cl_2Li_4N_5O_{12}P_3 \cdot 3H_2O$ requires C, 20.24; H, 2.76%); $\delta_P(D_2O$, pH 7) −10.16 (d, P_α, $^2J_{\alpha\beta}$ 18.2 Hz), +2.45 (dd, P_β, $^2J_{\alpha\beta}$ 28.2 Hz, $^2J_{\beta\gamma}$ 18.3 Hz), +8.55 (d, P_γ, $^2J_{\beta\gamma}$ 18.3 Hz); pK_a7.0 ± 0.1 (at 3 mM).

Using method (*b*) above, the bistriethylammonium salt was obtained as a white powder (251 mg, 23.3%); m.p. 230—240 °C (decomp.) (Found: C, 26.7; H, 5.3; Cl, 9.05; N, 10.6. $C_{11}H_{16}Cl_2N_5O_{12}P_3 \cdot 2C_6H_{15}N \cdot 6H_2O$ requires C, 26.05; H, 5.49; Cl, 9.07; N, 10.73%).

1,2-Ethynylbisphosphonic Acid Bis-tri-n-butylammonium Salt.—Tetramethyl 1,2-ethynylbisphosphonate[11] (0.52 g, 2.15 mmol) was stirred under a dry nitrogen atmosphere with bromotrimethylsilane (1.45 g, 9.45 mmol) at room temperature overnight, after which time a yellow solid was evident. The tetrasilyl ester ($\delta_P(CDCl_3)$ −30 p.p.m. (br) was not isolated but was dissolved in tetrachloromethane and hydrolysed with water. The aqueous phase was extracted with chloroform (2 × 10 ml) and evaporated to dryness to yield the free acid as a hygroscopic white solid (0.39 g, 98%); $\delta_P(D_2O)$ − 12.3 (s) [lit.,[12] $\delta(D_2O)$ −12.0 (s)]. The free acid (0.39 g, 2.11 mol) was dissolved in methanol (5 ml) and tri-n-butylamine added (0.83 g, 4.5 mmol). A mildly exothermic reaction ensued and the clear solution was stirred for a further 0.5 h. The solution was evaporated under reduced pressure to yield a white solid (1.24 g, 96.2% calculated as the bis salt), m.p. 130—135 °C (Found: C, 53.8; H, 10.05; N, 5.4. $C_2H_4O_6P_2 \cdot 2C_{12}H_{27}N \cdot H_2O$ requires C, 54.36; H, 10.45; N, 4.88%).

Adenosine 5'-(β,γ-μ-Ethynyl)triphosphate AMPPC≡CP (**1f**).—In a reaction analogous to the preparation of (**1c**) was used morpholine-4-*N*,*N*'-dicyclohexylcarboxamidinium adenosine-5'-phosphoromorpholidate (480 mg, 0.67 mmol) and tri-n-butylammonium 1,2-ethynylbisphosphonate (1.24 g, 2.03 mmol). The condensate was chromatographed on DEAE Sephadex using a linear salt gradient (LiCl, 0—0.5 M, pH 7.0, 2l) to give the *product* which was isolated by repeated dissolution in methanol (5 ml) and precipitation with acetone (25 ml) as a

white powder (227 mg, 55.4%), m.p. 210—230 °C (decomp.) (Found: C, 23.95; H, 3.6. $C_{12}H_{12}Li_4N_5O_{12}P_3 \cdot 4H_2O$ requires C, 23.57; H, 3.27%); $\delta_P(D_2O$ pH 9) −20.62 (dd, P_β, $^2J_{\alpha\beta}$ 22.5 Hz, $^3J_{\beta\gamma}$ 11.5 Hz), −11.4 (d, P_α, $^2J_{\alpha\beta}$ 22.5 Hz), and −10.26 (d, P_γ, $^3J_{\beta\gamma}$ 11.5 Hz).

Preparation of Guanosine 5′-(β,γ-μ-Difluoromethylenetriphosphate) GMPPCF$_2$P (**2b**)—Morpholine-4-*N*,*N*′-dicyclohexylcarboxamidinium guanosine-5′-phosphoromorpholidate (390 mg, 0.5 mmol) was dissolved in a mixture of anhydrous pyridine (5 ml) and freshly distilled 2-chlorophenol (4 ml).[13] To this was added the bis(tri-n-butylammonium) difluoromethylenebisphosphonate (580 mg, 1 mmol). The solution was stirred for 4 days with exclusion of moisture and light. After this time water (50 ml) was added and the solution extracted with ether (3 × 50 ml). The aqueous phase was evaporated to dryness and the gummy residue redissolved in water (5 ml), applied to a column of DEAE Sephadex, and eluted with a linear salt gradient (LiCl, 0—0.5 M, pH 7.0). Fractions containing the analogue were combined and evaporated to dryness and the product repeatedly precipitated from methanol with acetone. Evaporation to dryness of the final *product* in methanol yielded the title compound as a white powder (136 mg, 42.8%), m.p. 245—255 °C (decomp.) (Found: C, 21.2; H, 2.9; N, 10.3. $C_{11}H_{12}F_2Li_4N_5O_{13}P_3 \cdot 3H_2O$ requires C, 20.79; H, 2.84; N, 11.02%); $\delta_P(D_2O$ pH 9.0) −10.22 (d, P_α, $^2J_{\alpha\beta}$ 31.7 Hz), −1.80 (ddt, P_β, $^2J_{\alpha\beta}$ 31.7 Hz, $^2J_{\beta\gamma}$ 57.4 Hz, $^2J_{P\beta F}$ 89.1 Hz), and +4.50 (dt, P_γ, $^2J_{P\gamma F}$ 73.2 Hz, $^2J_{\beta\gamma}$ 57.4 Hz).

Guanosine 5′-(β,γ-μ-Fluoromethylene)triphosphate GMPPCFHP (**2c**).—In a reaction exactly analogous to that for (**2b**) morpholine-4-*N*,*N*′-dicyclohexylcarboxamidinium guanosine-5′-phosphoromorpholidate (144 mg, 0.2 mmol) was combined with the bis(tri-n-butylammonium) monofluoromethylenebisphosphonate (564 mg, 1 mmol), to yield the *product* as a white powder (76 mg, 63%); $\delta_P(D_2O$ pH 9.0) −10.34 (d, P_α, $^2J_{\alpha\beta}$ 27.4 Hz), +2.39 (ddd, P_β, $^2J_{\alpha\beta}$ 27.4 Hz, $^2J_{P\beta F}$ 62.8 Hz, $^2J_{\beta\gamma}$ 14.3 Hz), and +8.94 (dd, P_γ, $^2J_{\beta\gamma}$ 14.3 Hz, $^2J_{P\gamma F}$ 60.6 Hz).

Guanosine 5′(β,γ-μ-Dichloromethylene)triphosphate GMPPCCl$_2$P (**2d**).—In a reaction exactly analogous to that for (**2b**), morpholine-4-*N*,*N*′-dicyclohexylcarboxamidinium guanosine-5′-phosphoromorpholidate (144 mg, 0.2 mmol) was combined with the bis(tri-n-butylammonium) salt of dichloromethylenebisphosphonic acid[10] (360 mg, 0.6 mmol) to yield the *product* as a white powder (81 mg, 62%), m.p. 240—260 °C (decomp.) (Found: C, 18.8; H, 3.1; N, 9.35. $C_{11}H_{12}Cl_2Li_4N_5O_{13}P_3 \cdot 5H_2O$ requires C, 18.75; H, 3.12; N, 9.94%); $\delta_P(D_2O)$ −10.10 (d, P_α, $^2J_{\alpha\beta}$ 29.6 Hz), +2.10 (dd, P_β, $^2J_{\alpha\beta}$ 29.6 Hz, $^2J_{\beta\gamma}$ 17.6 Hz), and +8.80 (d, P_γ, $^2J_{\beta\gamma}$ 17.6 Hz).

Results and Discussion

Purity of Nucleotide Analogues.—All the ATP analogues were subjected to h.p.l.c. analysis at pH 4.5. Using a u.v. detection system at 254 nm they proved to be at least 99% pure. The AMP content was always less than 0.5% and the method of synthesis precluded the presence of ATP. The relative retention times were found to be AMP, 2.3 min; ADP, 3.3 min; ATP, 5.6 min; AMPPCF$_2$P, 5.15 min; AMPPCCl$_2$P, 4.45 min; AMPPCH$_2$P, 3.95 min; and AMPPC≡CP, 13.1 min. This appears to relate to the order of acidity of the ATP analogues.

The exception is the acetylene analogue (**1f**) whose retention time is much larger than anticipated. It is conceivable that this effect results from reversible covalent addition either of solvent or of buffer to the triple bond. This possibility opens the prospect of using acetylenic phosphonates as suicide substrates[14] for the inhibition of enzymes.

Table 1. ^{19}F N.m.r. chemical shifts (p.p.m.) and coupling constants (Hz) for nucleotide analogues

pH	Nucleotide	δ_F	$^2J_{P\beta F}$	$^2J_{P\gamma F}$
7.0	AMPPCF$_2$P (**1c**)	−118.6	89.5	70.2
7.0	AMPPCFHP[a] (**1d**)	−218	57	60
9.0	GMPPCF$_2$P (**2b**)	−119.5	89.1	73.2
9.0	GMPPCFHP[a] (**2c**)	−217	62.8	60.6

[a] Mixed diastereoisomers.

Assignment of Resonances.—The ^{19}F n.m.r. spectra at 94.17 Hz of the difluoromethylene analogues (**1c**) and (**2b**) showed a single, broad fluorine resonance for both nuclei. Unequal couplings to the adjacent phosphorus atoms were accurately determined from the ^{31}P n.m.r. spectra. Similarly the mixed diastereoisomers of the monofluoromethylene analogues (**1d**) and (**2c**) also showed a single broad resonance from which neither proton-fluorine nor phosphorus-fluorine coupling constants could be adequately derived (Table 1). While it is to be expected that the two resonances of the prochiral fluorines in AMPPCF$_2$P will diverge in the asymmetric environment of the enzyme-bound state, and thereby offer a useful probe of nucleotide fit, the coincidence of resonances in the diastereoisomeric mixture of AMPPCFHP is one of the factors which makes essential the separation of these two isomers prior to their use in enzyme studies.

The ^{31}P n.m.r. spectra of all the nucleotide triphosphate analogues prepared show typical AMX patterns. This is compounded with further coupling to fluorine nuclei for the four species (**1c,d**; **2b,c**) (Table 2). The characteristic downfield shift of some 20 p.p.m. typical of a phosphonic acid relative to the parent phosphate monoester along with analysis of coupling constants permits the direct assignment of resonances for P_α, P_β, and P_γ for all cases excepting the acetylene (**1f**). For this compound, the two doublets for P_α and P_γ are close together. However, the proton-coupled ^{31}P n.m.r. spectrum at 81 MHz shows that the upfield doublet (at pH 7.0) is proton-coupled (J_{PH} 5.5 Hz avg.). The assignment of this resonance to P_α is confirmed by its effective insensitivity to changes in pH (*vide infra* and Figure 2).

While the precise interpretation of phosphorus chemical shifts is subject to a complex set of factors,[15] it is greatly affected by the 'asymmetric loading' which is a summation of d_π orbital occupation, deviations from geometrical symmetry, and imbalance of σ-bonds from phosphorus to its substituents—which is a function of their electronegativity. Thus, variation in the paramagnetic shielding of the ^{31}P nucleus in phosphates and phosphonates results from changes in a 'tug-of-war' between the bonding electrons in four directions.

Utilising the fact that the P_α resonance is relatively invariant and the γ-phosphonate residue in the trinucleotide analogues should experience minimal geometrical perturbation, the downfield shift change, $\Delta(\gamma - \alpha)$, would provide a measure of the balance between electronegativity and d_π-bonding effects at P_γ (Table 2). This downfield shift difference generally increases with diminishing electronegativity of the P_β–X–P_γ bridging group in the series O > CF_2* > NH > CFH ≈ CCl_2 > CH_2 in the sense that increased ligand electronegativity gives *increased* magnetic shielding at P_γ. For those ligands, notably oxygen and nitrogen, capable of d_π-bonding to phosphorus it is apparent that there is an additional shielding effect (Table 2).

* Cavell has shown that the element effect of CF_3 and F as ligands for phosphorus are very similar.[18]

Table 2. ^{31}P N.m.r. chemical shifts (p.p.m.) and coupling constants (Hz) for nucleotide analogue

pH	Nucleotide	δ_α	δ_β	δ_γ	$^2J_{\alpha\beta}$	$^2J_{\beta\gamma}$	$\Delta_{(\beta-\alpha)}$	$\Delta_{(\gamma-\alpha)}$
10.0	ATP (**1a**)	−10.2	−20.7	−5.0	18.8	19.5	−10.5	+5.2
10.0	AMPPNHP (**1b**)	−9.66	−6.15	+0.32	20.75	5.5	+3.5	+10.0
10.0	AMPPCF$_2$P (**1c**)	−10.37	−2.2	+4.27	32.6	58.0	+8.2	+14.6
7.0	AMPPCFHP[b] (**1d**)	−10.48	+2.42	+8.58	29.0	15.0	+12.9	+19.6
9.0	AMPPCCl$_2$P (**1e**)	−10.16	+2.45	+8.55	28.2	18.3	+12.6	+18.8
9.0	AMPPC≡CP (**1f**)	−11.40	−20.62	−10.26	22.5	11.5[a]	−9.2	+1.1
10.0	AMPPOOP[c] (**1g**)	−12.10	−9.9	+5.7	21	13	+2.2	+17.8
10.0	AMPPCH$_2$P (**1h**)	−10.35	+14.36	+12.14	26.85	7.9	+24.7	+22.5
9.0	GMPPCF$_2$P (**2b**)	−10.22	−1.80	+4.50	31.7	57.3	+8.4	+14.7
9.0	GMPPCFHP[b] (**2c**)	−10.34	+2.39	+8.94	27.4	14.3	+12.7	+19.3
9.0	GMPPCCl$_2$P (**2d**)	−10.10	+2.10	+8.80	29.6	17.6	+12.2	+18.9

[a] $^3J_{\beta\gamma}$. [b] Mixed diastereoisomers. [c] Ref. 16.

Two of the analogues deserve individual comment. The acetylenic phosphonate (**1f**) shows the smallest downfield shift, $\Delta(\gamma - \alpha)$, of all the compounds studied. This can be attributed to the combined effects of greater electronegativity for sp-relative to sp^3-hybridised carbon, of possible d_π-bonding to phosphorus, and of the characteristically strong, diamagnetic anisotropic shielding by the acetylenic function along the carbon axis.[19] Leonard's peroxyphosphonate[16] (**1g**) exhibits a larger downfield shift difference, $\Delta(\gamma - \alpha)$, than the imino (**1b**) or difluoromethylene (**1c**) analogues and its +12 p.p.m. shift difference for P_γ relative to ATP is an anomaly not simply to be explained by electronegativity and d_π-bond effects.

The resonances for P_β are downfield from P_α for all analogues except the acetylene (**1f**) and ATP itself. This behaviour is typical for internal phosphorus residues in poly- and meta-phosphate systems[20] and lies outside that range of phosphorus chemical shifts which can be shown to depend on bond angle effects[21] or torsional angle conformations[22] for phosphate diesters. For the present study of nucleotide analogues, it will serve to note that the 35 p.p.m. difference in chemical shift for P_β between ATP and AMPPCH$_2$P accommodates a range which reflects the combined electronegativity and d_π-bonding possibilities for a P_β ligand. It can be seen in this respect that CF_2 is a little inferior to NH, while CFH and CCl_2 lie about halfway between CF_2 and CH_2. As before, the acetylenic group in (**1f**) fully mimics ATP while the peroxide (**1g**) exhibits an 11 p.p.m. downfield shift for P_β.

Coupling Constants and Conformation.—The vicinal proton-proton coupling constants for the ribose ring were obtained by analysis of spectra at 400 MHz. These gave first-order patterns for 1′-H, 2′-H, and 3′-H (Table 3). The established derivation[23] of ribose ring conformation from the ratio $J_{1'2'}/J_{3'4'}$ shows that for all the analogues the preferred sugar pucker is C2′-*endo* and ranges from 55% ^2E for ATP to 70% ^2E for AMPPC≡CP. The signals for 4′-H, 5′-H, and 5″-H were observed as an unresolved multiplet and coupling constants could not be derived from them. However, $^{31}P_\alpha$ spectra provided values* for $^3J_{PH^5}$ of *ca.* 5.5 Hz which indicates that the C(5′)–O(P) bond adopts the normal *trans* conformation, Φ_a.[23]

The $^2J_{PF}$ coupling constants (Table 1) show a spread of 30 Hz and are assumed to be positive[19] though no attempt was made to determine their sign. If their magnitude is strongly distance dependent, as is the case for $^2J_{HF}$ couplings,[24] they indicate that the difluoromethylenephosphonates exist in a more compact structure than do the monofluoro analogues.

* The $^{31}P_\alpha$ signal appeared as a double triplet in the proton-coupled mode.

Table 3. Pentose conformational analysis of nucleotide analogues by ^1H n.m.r. coupling constants (Hz)

Nucleotide	$^3J_{1'2'}$	$^3J_{3'4'}$	$J_{1'2'}/J_{3'4'} = {}^2E/{}^3E$	^2E (%)
ATP	5.3	4.5	1.18	55
AMPPCCl$_2$P	5.8	4.6	1.26	56
GMPPCCl$_2$P	6.0	3.7	1.62	62
AMPPCF$_2$P	6.0	3.6	1.67	62
GMPPCF$_2$P	6.1	3.5	1.74	63
GMPPCFHP	6.0	3.2	1.87	65
AMPPC≡CP	6.6	2.8	2.36	70

The phosphorus-phosphorus coupling, $^2J_{\alpha\beta}$ for nucleoside triphosphates is usually close to 20 Hz, a value which is typical for $^2J_{PP}$ in condensed phosphates.[20] The present data show that this figure is susceptible to a 50% increase as a result of changes in the substituents at P_β though these changes do not appear to correlate with the electronegativity of the variable P_β substituent.

The $^2J_{\beta\gamma}$ couplings for phosphorus show a much larger spread and deviate both above and below the 20 Hz value typical for trinucleotides.[15] Two values stand apart from the rest: those for the difluoro analogues (**1c**) and (**2b**). The best interpretation seems to be that they signal a marked conformational change in the phosphoryl chain that is not manifest for the dichloro- or monofluoro-methylene species. This possibility might be open to further examination by n.m.r. studies on protein-bound nucleotide analogues.

Acid Dissociation Constants.—The fourth dissociation constants for the ATP analogues were determined by titration of solutions of the tetralithium salts (3—10 mM) from pH 3.5 with standard alkali. The results (Table 4) show the typical reduced acidity of phosphonic acids relative to phosphate monoesters.[5] This effect is more than adequately overcome by the enhanced electronegativity of the CCl_2 and CF_2 groups which impart to the nucleotide analogues that enhancement of acidity seen in the parent halomethylenebisphosphonic acids.[25] In particular, the phosphonates (**1c**), (**1e**), (**2b**), and (**2d**) have the biologically important tetra-anion stability markedly increased at physiological pH.

In the case of the acetylenic analogue (**1f**) the value of the dissociation constant was derived from analysis of the ^{31}P n.m.r. chemical shifts of the β- and γ-phosphorus resonances as a function of pH, giving $pK_a^4 = 5.5 \pm 0.1$. This value is lower than expected and may indicate that in the majority of ATP species studied there is hydrogen bonding from P_γ–O–H to P_β–O, which is precluded in (**1f**) by the linear acetylenic linkage.

Divalent Metal Ion Binding.—The binding of divalent metal ions to the ATP analogues was investigated by two methods:

Table 4. Dissociation constants for proton and metal cation binding to nucleotides at 25 °C

Nucleotide	pK_a^4	pK_a^a Ca^{2+}	pK_a^a Ba^{2+}
ATP (**1a**)	7.1	4.7	4.6
AMPPNHP (**1b**)	7.7	4.6	
$AMPPCF_2P$ (**1c**)	6.7	4.3	4.0
$AMPPCCl_2P$ (**1e**)	7.0	4.6	5.3
AMPPC≡CP (**1f**)	5.5[b]	3.3	4.8
$AMPPCH_2P$ (**1h**)	8.4	4.2	

[a] Metal binding at pH 8.5, [Nucleotide] = 0.5 to 1.0 mM. [b] Value obtained from ^{31}P n.m.r. data (Figure 2).

direct evaluation using ion-sensitive electrodes and changes in ^{31}P n.m.r. chemical shifts resulting from metal complex formation.

The appropriate ion-selective electrodes were employed to determine the free calcium and barium ion concentration in 1:1 metal–nucleotide complex mixtures in solution at 25 °C. (Attempts to use a 'hardness of water' electrode for the same purpose for magnesium complexes was unsuccessful.) pK_d Values were calculated directly and the average taken of three or more separate determinations (Table 4). The value determined in this way for the binding of calcium to ATP, $pK_d = 4.7$, lies just beyond the wide range of values, from 3.3 to 4.5, which have been determined by several, mainly indirect, methods.[26,27] The present direct method gives very reproducible results which are of more than sufficient accuracy to use for comparisons between the various ATP analogues.

A factor of 3 covers the dissociation constants for calcium binding of all analogues except for the acetylene (**1f**). The latter binds calcium 25 times more weakly than does ATP. This result clearly shows that calcium requires bidentate chelation to the β- and γ-phosphate residues of ATP in a mode that is not very dependent on small changes in the angle of the P_β–P_γ bridge but which is effectively inhibited by the linear acetylenic bridge. It is clearly desirable that further information be obtained from studies using the α,β-acetylene ATP analogue, AMPC≡CPP, which should permit β,γ- but not α,β-chelation.

Per contra, the β,γ-acetylene (**1f**) complexes barium as well as does ATP. Since the O_β:O_γ separation is some 5.5 Å (from molecular models) it appears that barium is able to chelate equally well to the α,β-oxygens.

The unusually high perceived binding of barium to the dichloromethylene analogue (**1e**) demanded measurements at the lower limit of linear response of the barium-sensitive electrode and may thus be of lower accuracy than the other values of pK_d given. Binding of this strength suggests that there may be a sterically-enforced conformational change in the phosphate chain imposed by the two chlorine atoms. This view is possibly supported by the n.m.r. observation that $AMPPCCl_2P$ shows none of the line-broadening for 8-H relative to 2-H which is characteristic of spectra of the other halogenemethylene analogues and is attributable to through-space effects from the phosphate chain.

^{31}P N.m.r. spectroscopy has been used widely to investigate divalent metal binding to ATP and its analogues.[28–31] In the present work we have used two different methods.

Firstly, the change in chemical shift for P_α, P_β, and P_γ, was monitored before and after the addition of 1.1 equivalents of Mg^{2+}, Ca^{2+}, or Zn^{2+} to the nucleotide analogue at pH 9.15 (Table 5). The barium complex with ATP was insoluble above 2 mM. These data reflect changes in phosphorus shielding on formation of 1:1 metal complexes with the tetra-anions. For some analogues, the corresponding changes were also monitored at lower pH for complex formation with trianions.

For the tetra-anion of ATP, all three phosphorus resonances move downfield following the formation of the 1:1 magnesium complex. For all the metals used, this change is much the largest for P_β. For the imino- and methylene-analogues, (**1b**) and (**1h**), the downfield shift for P_β is greatly reduced while the shift for P_γ is now upfield. In the case of the halogenomethylene analogues, (**1c**), (**1d**), and (**2b**), all three resonances exhibit upfield shifts on forming 1:1 complexes with magnesium, calcium, and zinc cations. Finally, the acetylenic analogue (**1f**) shows chemical shifts independent of the addition of magnesium, which indicates that there is no significant complex formation under the conditions used.

These apparently quixotic results actually reveal whether the metal complexes are better analogues than the non-complexed salts relative to ATP. The *difference* in the effect of complexation of metal with ATP and the analogue ($\Delta\delta_{\alpha ATP} - \Delta\delta_{\alpha\ analogue}$) is an upfield shift in every case for P_α, P_β, and P_γ. Moreover, the magnitude of this upfield effect is about twice as big for the dihalogenomethylene ATP analogues, (**1c**) and (**1e**), as for AMPPNHP and $AMPPCH_2P$. As gauged by the ^{31}P n.m.r. criterion, halogenation of the methylene phosphonates improves their analogy to ATP even more in the M^{II} complexes than in the free phosphonates.

The second type of analysis was employed only for the difluoromethylene and acetylenic analogues of ATP. ^{31}P N.m.r. spectra were scanned as a function of pH for the analogues alone and in the presence of 1.1 equivalents of magnesium chloride. The titration curves are accurately fitted for a pK_a^4 of 6.69 ± 0.05 for $AMPPCF_2P$ and of 5.5 ± 0.1 for AMPPC≡CP while the magnesium complex of the former has pK_a^4 = 5.2 ± 0.1 (Figures 1 and 2). Similar reductions in pK_a^4 have been observed for magnesium complex formation with ATP,[29,32] AMPPNHP,[28] and $AMPPCH_2P$.[33,34] By contrast, the titration curve for AMPPC≡CP is unchanged in the addition of 10mM-$MgCl_2$ which suggests that pK_d for magnesium complex formation with (**1f**) is less than *ca.* 3.5.

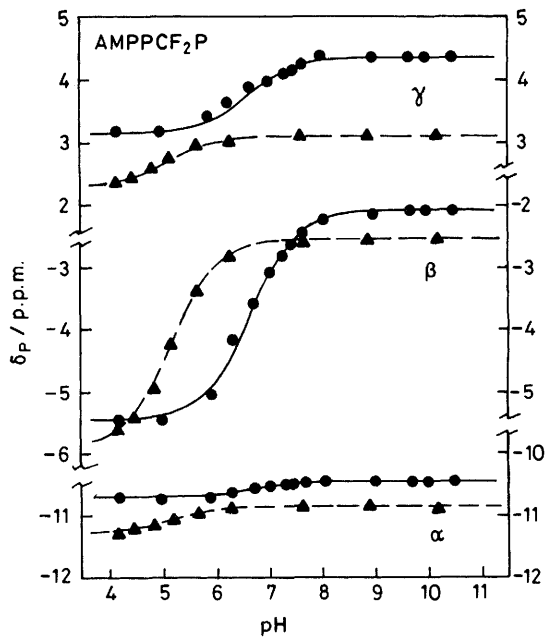


Figure 1. ^{31}P N.m.r. chemical shifts for $AMPPCF_2P$, (**1c**), as a function of pH in the presence (▲) and absence (●) of 1.1 equivalents of $MgCl_2$. Chemical shifts (p.p.m.) downfield from external 85% H_3PO_4 are positive. Substrative (13.3 mM) dissolved in 30% D_2O with 50 μM EDTA. Calculated curves for pK_a = 6.68 (-●-) and 5.20 (-▲-).

Table 5. Effect of divalent metal ion binding on ^{31}P n.m.r. chemical shifts (p.p.m.) of nucleotides. (Downfield shifts after adding M^{2+} are positive)

Nucleotide*	Metal ion	pH	$\Delta\delta_\alpha$	$\Delta\delta_\beta$	$\Delta\delta_\gamma$	ΔpK_a[4]
ATP	Mg^{2+}	9.15	0.5	3.9	0.6	−1.4[a], −1.55[b]
	Mg^{2+}	3.3[b]	0.5	3.7	0.5	
	Ca^{2+}	9.15	0.35	2.4	0.7	
	Zn^{2+}	9.15	0.3	2.6	0.0	
	Ba^{2+}[c]	7.5	0.3	1.2	0.5	
$AMPPCF_2P$	Mg^{2+}	9.15	−0.4	−0.5	−1.3	−1.5
	Mg^{2+}	3.5	−0.55	−0.35	−0.8	
	Ca^{2+}	9.15	−0.03	−0.8	−1.25	
	Zn^{2+}	9.15	−0.33	−0.9	−0.2	
$AMPPCCl_2P$	Mg^{2+}	9.15	0	−1.1	−2.15	
	Ca^{2+}	9.15	−0.03	−0.95	−1.7	
	Zn^{2+}	9.15	−0.15	−0.55	−0.4	
$AMPPCH_2P$[a,e]	Mg^{2+}	10.0	+0.3	+1.2	−0.3	−1.8[d], −2.4[e]
	Mg^{2+}	4.0	+0.35	+1.05	−0.4	
AMPPNHP[f]	Mg^{2+}	10.0	0.15	1.1	−0.8	−1.5[f]
	Mg^{2+}	6.0	0	1.0	−0.9	
AMPPC≡CP	Mg^{2+}	9.15	0.15	<0.1	<0.1	0
		3.5	0.1	<0.1	<0.1	
$GMPPCF_2P$	Mg^{2+}	9.15	−1.13	−1.0	−1.55	
	Ca^{2+}	9.15	−0.03	−0.8	−1.3	
	Zn^{2+}	9.15	+0.3	−1.0	+0.1	

* Nucleotide = 10 mM ± 10 mM MCl_2.
[a] Ref. 29. [b] Ref. 32. [c] [complex] *ca.* 2 mM. [d] Ref. 33. [e] Ref. 34. [f] Ref. 28.

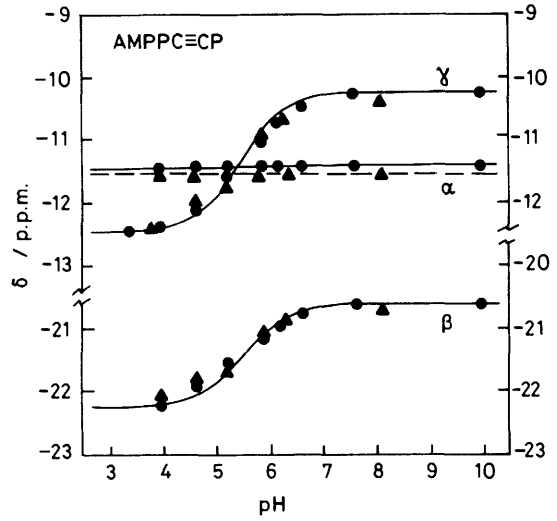


Figure 2. ^{31}P N.m.r. chemical shifts for AMPPC≡CP as a function of pH in the presence (▲) and absence (●) of 1.1 equivalents of $MgCl_2$. Sample details as for Figure 1. Calculated curves for pK_a = 5.50.

Early studies of the effect of divalent metal ion complex formation supported the conclusion that the chemical shift data obtained with non-paramagnetic metals lends itself to interpretation in terms of molecular structure.[35] Later workers concluded that magnesium binds to ATP through the β-phosphate and not through the α- and γ-phosphates.[32] This simplification has been critically re-judged in the light of more recent ^{31}P n.m.r. results. It is now thought that effects exerted by magnesium and calcium are due mainly to changes in conformation of the polyphosphate chain of ATP rather than to electronic factors associated with the binding of divalent cations to specific oxy-anions.[30,33] The situation is complicated by the fact that magnesium binding is very probably observed as the time-average, fast-exchange of all possible metal-co-ordination types[33] and it has been argued[31] that the 1:1 ATP^{4-} complex has Mg^{2+} bound in a tridentate fashion to non-bridging oxygens at P_α, P_β, and P_γ. A recent ^{17}O n.m.r. study suggests that there is lesser co-ordination to P_α in ATP than to P_β or P_γ but that such co-ordination must be related only to the 'macroscopic' structure of MgATP and not to its 'microscopic' structure.[36]

From the present study, it is clear that tight magnesium and calcium binding to ATP and its β,γ-analogues requires β,γ-co-ordination since the linear acetylenic analogue, AMPPC≡CP, fails to match the strength of metal binding to all other species. It seems likely that the as yet unknown α,β-acetylene analogue, AMPC≡CPP, should provide a good test of whether β,γ-co-cordination is sufficient when it cannot be augmented by simultaneous cation binding to the P_α-oxygen anion.

It is equally apparent that the strength of metal binding to a particular P–O anion is not reflected in the magnitude of ^{31}P n.m.r. shift change since for some analogues, notably $AMPPCH_2P$ and AMPPNHP, the shift changes even reverse direction! However, the performance of $AMPPCF_2P$ and $AMPPCCl_2P$ on complexation with magnesium and calcium shows that good approximation to the behaviour of ATP *can* be registered by such isopolar and isosteric analogues of ATP.

These two compounds have performed more than adequately in the evaluation of their physical constants and thus merit proper testing as substrate analogues and as inhibitors in ATP-utilising enzyme systems. Equally, the existence of AMPPC≡CP as a tetra-ionic analogue of ATP which does not bind magnesium or calcium may prove to have particular biological applications.

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