Experimental Section

2a: To a solution of **1a** (53.2 mg, 0.092 mmol) in CH_2Cl_2 (10 mL) was added tcne (13 mg, 0.10 mmol) at room temperature. After 4 h the solvent was removed, and the solids were washed with diethyl ether. The residue was crystallized from CH_2Cl_2 and diethyl ether to give orange-brown crystals of **2a** (43.2 mg, 66.3 %). IR (nujol): $\tilde{\nu} = 2250\ cm^{-1}$ (C≡N); UV/Vis (CH_2Cl_2): $\lambda_{max} = 392$, 329 nm; ^1H NMR (250 MHz, $CDCl_3$): $\delta = 1.50$ (d, $J(P,H) = 5.0$ Hz, Cp*), 3.48 (s, OMe), 5.27 (s, CH_2Cl_2), 6.36 (s, CH), ca. 6.06 and 7.3 – 8.0 (m, ArH); ^{31}P NMR (100 MHz, $CDCl_3$): $\delta = 45.2$ (d, $J(Rh,P) = 142.7$ Hz); FAB-MS: m/z: 709 [M^+], 673 [$M^+ - Cl$]; elemental analysis calcd for $C_{35}H_{31}N_4O_2PClRh \cdot CH_2Cl_2$: C 54.46, H 4.19, N 7.06; found: C 54.87, H 4.08, N 6.84.

2b: Yellow crystals of **2b** (41.4 mg, 42.9 %) were obtained from **1b** (80 mg, 0.121 mmol) and tcne (21.8 mg, 0.170 mmol) by a precedure similar to that for **2a**. UV/Vis (CH_2Cl_2): $\lambda_{max} = 324$ nm; ^1H NMR (250 MHz, $CDCl_3$): $\delta = 1.53$ (s, Cp*), 3.50 (s, OMe), 5.24 (s, CH_2Cl_2), 6.31 (s, $HC(CN)_2$), ca. 6.13 and 7.2 – 7.9 (m, ArH); ^{31}P NMR(100 MHz, $CDCl_3$): $\delta = 26.2$ (s); FAB-MS: m/z: 798 [M^+]; elemental analysis calcd for $C_{35}H_{31}N_4O_2PClIr$: C 52.66, H 3.91, N 7.02; found: C 52.98, H 3.84, N 7.00. Crystal data: $C_{35}H_{31}N_4O_2PClIr$, monoclinic, space group $P2_1/n$ (no. 14), $a = 12.123(5)$, $b = 14.314(7)$, $c = 20.407(3)$ Å, $\beta = 95.34(2)^\circ$, $V = 3525(1)$ Å^3, $Z = 4$, $\rho_{calcd} = 1.504\ g\,cm^{-3}$, $R = 0.028$ and $Rw = 0.037$ [$w = 1/\sigma^2(F_o)$] for 4487 reflections [$I > 3.0\sigma(I)$] with 397 variables. The structure was solved by Patterson methods (DIRDIF92) and refined by full-matrix least-squares techniques using the teXsan program package.[10]

3a: Orange crystals of **3a** (28 mg, 32 %) were obtained from **1a** (60 mg, 0.103 mmol) and tcnq (25 mg, 0.13 mol) by a precedure similar to that for **2a**. IR (nujol): $\tilde{\nu} = 2247\ cm^{-1}$ (C≡N); UV/Vis (CH_2Cl_2): $\lambda_{max} = 398$, 330 nm; ^1H NMR (250 MHz, $CDCl_3$): $\delta = 1.35$ (d, $J(P,H) = 3.0$ Hz, Cp*), 3.44 (s, OMe), 5.08 (s, CH), 7.51 (AB q, $J = 10.0$ Hz), ca. 6.00 and 7.3 – 8.0 (m, ArH); ^{31}P NMR (100 MHz, $CDCl_3$): $\delta = 8.4$ (d, $J(Rh,P) = 150.0$ Hz); FAB-MS: m/z: 785 [M^+], 750 [$M^+ - Cl$]; elemental analysis calcd for $C_{41}H_{35}N_4O_2PClRh$: C 62.73, H 4.49, N 7.14; found: C 62.55, H 4.55, N 7.29. Crystal data: $C_{41}H_{35}N_4O_2PClRh$, monoclinic, space group $P2_1/n$ (no. 14), $a = 13.70(1)$, $b = 19.076(7)$, $c = 15.965(10)$ Å, $\beta = 101.31(6)^\circ$, $V = 4092(4)$ Å^3, $Z = 4$, $\rho_{calcd} = 1.274\ g\,cm^{-3}$, $R = 0.055$ and $Rw = 0.080$ for 2961 reflections [$I > 4.0\sigma(I)$] with 451 variables. The structure was solved by Patterson methods (DIRDIF92) and refined by full-matrix least-squares techniques using the teXsan program package.[10]

Received: September 14, 1998
Revised version: January 15, 1999 [Z 12409 IE]
German version: *Angew. Chem.* **1999**, *111*, 1318 – 1320

Keywords: C–H activation · insertions · iridium · olefins · rhodium

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New Tripodal, "Supercharged" Analogues of Adenosine Nucleotides: Inhibitors for the Fhit Ap_3A Hydrolase**

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Diadenosine polyphosphates, discovered over 30 years ago,[1] are ubiquitous components of all cells. Recently, diadenosine triphosphate and tetraphosphate, Ap_3A and Ap_4A, have assumed vital significance as ligands for the tumor suppressor protein, Fhit,[2a] which is an Ap_3A hydrolase[2b] whose signalling appears to depend on Ap_nA binding.[3] We are interested in the specific enzymes of dinucleoside polyphosphate catabolism,[2a, 4, 5] in the chemistry[6] of those compounds, and also in bisubstrate analogues for phosphoglycerate[7] and other kinases, and present here the synthesis

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[**] This work was supported by the BBSRC (ROPA/MOLO4558 and B08013), by the KBN (Project 6P04A 062 15), and by the U.S. National Cancer Institute (CA75954). Overexpressed (asymmetrical) Ap_4A hydrolases from lupine and human were generous gifts from Dr. K. R. Gayler (Melbourne University, Australia) and Dr. A. G. McLennan (Liverpool University, UK), respectively. We thank Mr. Peter Ashton (Birmingham University) for assistance with high-resolution MS analyses.

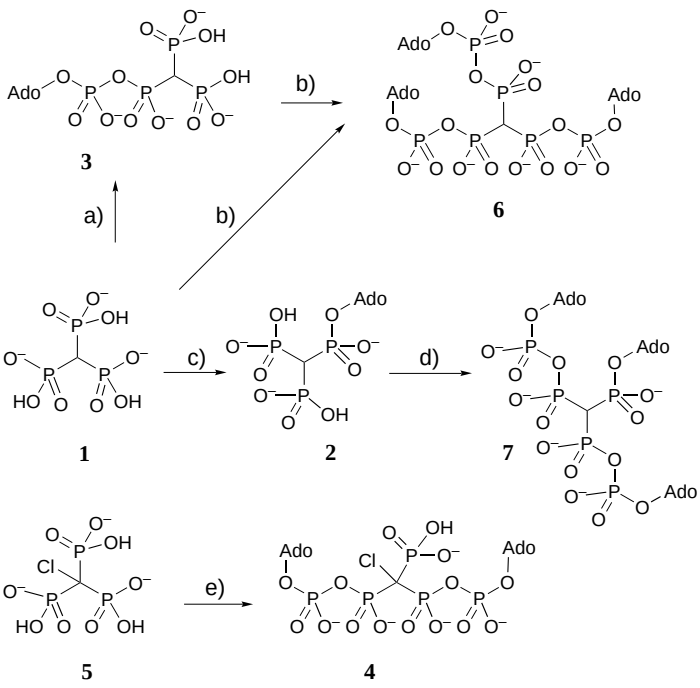
  1433-7851/99/3809-1244 $ 17.50+.50/0

of novel bisubstrate analogues that are isopolar[8] to the transition state for Ap_4A hydrolysis.

A key problem in these endeavors is the unavoidable diminution in anionic charge of a linear bisubstrate nucleotide analogue relative to that in the transition state for the corresponding phosphoryl transfer reaction. For example, adenylate kinase (AK) interconverts two ADP molecules (net substrate charge -6) with AMP plus ATP via a trigonal-bipyramid transition state of charge -6. The bisubstrate analogue for this reaction, Ap_4A, has a charge of only -4. Not surprisingly, Ap_5A ($K_i = 30$ nM) is a superior inhibitor of AK compared to Ap_4A ($K_i = 10$ μM),[9] though we note that phosphate chain conformational factors may also contribute to this difference. Likewise, the lupine Ap_4A hydrolase cleaves Ap_4A to give AMP plus ATP via an in-line trigonal-bipyramid transition state[10] of charge -5. There is thus a good opportunity to generate improved bisubstrate analogue inhibitors for many phosphoryl transfer enzymes through the use of pyrophosphate mimics that incorporate additional negative charge by branching rather than by phosph(on)ate chain elongation. Previously we have described the synthesis of some such "supercharged" analogues of pyrophosphoric acid (**1**, **5**) and their incorporation into analogues of ATP.[11] We now report their incorporation into analogues of ADP and of diadenosine polyphosphates and especially the novel use of the C_3 symmetry branch point of methanetrisphosphonic acids to create new tripodal adenosine nucleotide analogues.

These products show differential competitive inhibition between two diadenosine triphosphate hydrolases. Ap_nA hydrolases appear to comprise at least two evolutionary groups. The human *fhit* gene product is a member of the histidine triad superfamily[12a] of nucleotide binding proteins which has homologues in animals and fungi. Other Ap_nA hydrolases are related to mutT[12b] and have been found in all forms of life. Because the Fhit protein is a tumor suppressor protein that is inactivated early in the development of epithelial cancers[12c] but is not the only enzyme that can cleave Ap_3A, new chemical entities that distinguish between Fhit and other Ap_nA hydrolases have great potential in cancer diagnostics.

Methanetrisphosphonic acid (**1**) was described by Gross et al. in 1993.[13] Key improvements in that synthesis have enabled us now to prepare chloro- and fluoromethanetrisphosphonic acids for the first time and in usable quantity.[14] Compound **1** was readily transformed into an ADP analogue (**2**) by the method of Poulter et al.[15] and into an analogue of ATP (**3**) by the phosphoromorpholidate procedure of Khorana and Moffatt[16] (Scheme 1). For ATP analogue synthesis[11] AMP morpholidate was used as the limiting reagent, while in the case of diadenosine tetraphosphate analogue **4**, prepared from chloromethanetrisphosphonate (**5**), AMP morpholidate was used in excess. The *two* primary phosphonic acid functionalities in the ADP analogue **2** and the ATP analogue **3** were sites for double adenylation by a modification to the procedure of Khorana and Moffatt in the synthesis of two novel tripodal nucleotide analogues (Scheme 1). The reaction of the tris-tri-*n*-butylammonium salt of **1** with AMP morpholidate was extremely sluggish, and only a small amount of the desired trisadenylylated product **6** was obtained after seven



Scheme 1. Synthesis of the nucleotide analogues **2**–**7**. a) AMP-morpholidate (0.8 equiv), tetrazole, pyridine; b) AMP-morpholidate (5 equiv), tetrazole, pyridine; c) 5′-*O*-tosyl adenosine, MeCN; d) excess AMP-morpholidate, pyridine; e) AMP-morpholidate (2.2 equiv), tetrazole, pyridine. All counterions are tri-*n*-butylammonium. Ado = 5′-adenosyl.

days. Evidently the incorporation of the third adenylate moiety was extremely slow since both the intermediate ATP analogue **3** and the Ap_4A analogue **4** (with H instead of Cl) were isolated as major products. Tetrazole proved to be an excellent catalyst,[17] and reaction was complete in two days to give P^1,P^2,P^3-tris(5′-adenylyl) methanetrisphosphonate (**6**) in 59% yield. Similarly, 5′-adenosyl methanetrisphosphonate (**2**) was treated with an excess of AMP morpholidate with tetrazole catalysis to give the tripodal nucleotide analogue P^1-5′-adenosyl P^2,P^3-bis(5′-adenylyl) methanetrisphosphonate (**7**) in 58% yield. These provide the first two examples of three adenylate moieties linked together by a methanetrisphosphonate core.[18] The pK_a values for these species were determined and clearly show the benefit of α-halogenation for increasing the acidity of the deprotonated phosphonic acids (Table 1).

We envisaged that these novel nucleotide analogues might be substrates for Ap_nA hydrolases, which cleave the P^1,P^2-pyrophosphate linkage in Ap_nAs.[5, 19] Assays with two *asymmetrical* Ap_4A hydrolases (EC 3.6.1.17), one from lupine[4] and a recombinant human Ap_4A hydrolase,[20] showed that none of the nucleotide analogues behaved as substrates while they are all strong, nondiscriminatory inhibitors of these Ap_4A hydrolases (data not presented). However, these analogues did not inhibit the activity of a lupine adenosine 5′-tetraphosphate hydrolase.[21] We then examined members of two different classes of Ap_3A hydrolase (EC 3.6.1.29), Fhit protein[2b] and an Ap_3A hydrolase from yellow lupine seeds.[22] The test nucleotides were not hydrolyzed, and we now found that they inhibited these two enzymes in very contrasting ways (Figure 1).

  1433-7851/99/3809-1245 $ 17.50+.50/0

Table 1. The pK_a values, inhibition constants, and net charges calculated for inhibition of human Fhit and yellow lupine Ap_3A hydrolases by a range of methanetrisphosphonates and their adenosine nucleotide derivatives.[a]

Inhibitor	pK_a	Human Fhit Ap_3A hydrolase		Lupine Ap_3A hydrolase		δ_P
		K_i [μM]	net charge pH 6.9	K_i [μM]	net charge pH 8.2	D_2O, pD 7
PCF(P)P (**5**, F instead of Cl)	p$K_{a4,5}$ 5.77, 8.86	n.d.	3.93	1.0	4.18	8.89; s
PCCl(P)P (**5**)	p$K_{a4,5}$ 5.92, 9.08	n.d.	3.91	1.0	4.12	11.42; s
PCH(P)P (**1**)	p$K_{a4,5}$ 6.46, 9.90	n.d.	3.74	1.3	3.98	13.17; s
AdoPCH(P)P (**2**)	pK_{a4} 7.48	18.3	3.21	6.1	3.84	15.15, 12.94; ABB′
AdoPPCH(P)P (**3**)	pK_{a5} 7.50	4.0	4.20	2.8	4.83	13.23, 7.85, −9.41; AA′MX[11]
AdoPPCCl(P)PPAdo (**4**)	pK_{a6} 8.86	1.9	5.01	16.2	5.18	8.18, 3.75, 1.36, −9.33; AA′MM′X
AdoPCH(PPAdo)$_2$ (**7**)	n.a.	1.2	5.00	3.0	5.00	13.48, 5.08, −9.78; AA′MM′X
(AdoPP)$_3$CH (**6**)	n.a.	0.4	6.00	5.4	6.00	5.22, −9.46; AA′A“XX′X”

[a] The pK_a values were measured in the range $3.5 < pH < 10.5$. n.a.: no ionization change in this pH range. n.d.: no inhibition detectable at an inhibitor concentration of 200 μM or less. Each enzyme was assayed at the pH at which it shows maximum activity.

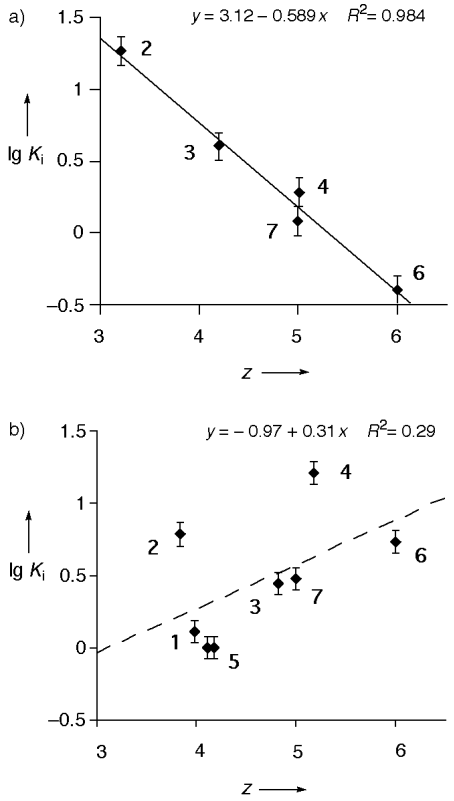


Figure 1. Linear free energy plots of lg K_i versus inhibitor charge z for the binding of a range of trisphosphonates and their nucleotide analogues to Ap_3A hydrolases from human Fhit (a) and yellow lupine sources (b). The K_i values are in μM. Charge is calculated from pK_a values (Table 1) respective to the pH optimum for enzyme activity: a) Fhit, pH 6.9; b) lupine Ap_3A hydrolase, pH 8.2.

Methanetrisphosphonate (**1**) and its chloro (**5**) and fluoro derivatives (**5**, F instead of Cl) do not detectably inhibit human Fhit, but are strong inhibitors of the lupine enzyme with K_i values similar to the K_m value for Ap_3A (1.2 μM, Table 1).[23] By contrast, most of the adenylated polyphosph(on)ates strongly and competitively inhibit Fhit with K_i values similar[3b] to the K_m value for Ap_3A (1.9 μM) while they are less effective as inhibitors of the lupine enzyme (Table 1). Most of the difference in inhibition constants for these two hydrolases evidently stems from contrasting charge dependencies of ligand binding. The competitive inhibition of Fhit shows a rather good linear free energy relationship to the anionic charge for five adenosine phosphonate analogues (calculated at the pH optimum of the enzyme assay), providing that at least one adenosine moiety is available for binding (Figure 1a). That requirement accords with results from X-ray crystal structures of two diastereoisomers of 5′,5‴-diadenosyl P^1-thio-P^2,P^3-methylenetrisphosphate complexed to a wild-type and to an active-site mutant of Fhit. These both show[3b] one adenosine in a deep cleft while the second is in surface contact with bulk solvent. By contrast, the strongest inhibitors of the lupine enzyme are the three methanetrisphosphonic acids lacking any adenosine moiety. Moreover, increasing the charge on the inhibitors broadly tends to a decrease in affinity for the lupine enzyme, though the correlation is weak (Figure 1b).

These novel tripodal adenine nucleotides have provided a first example of differential inhibition of human Fhit from an alternative Ap_3A hydrolase, with a better than tenfold selectivity ratio (Table 1). Detection of levels of Fhit protein is an important cancer problem that currently is complicated by the activity of unrelated enzymes that cleave Ap_3A. For this reason, Fhit-selective inhibitors such as **4** and **6** will be valuable as Fhit diagnostics, while the greater than 1000-fold selectivity for competitive inhibition of the lupine enzyme by the non-nucleotide species **1** and **5** is expected to fulfill a complementary role in inhibiting lupine-related Ap_3A hydrolytic activities in human cells. This differential selectivity could provide a valuable tool for further study of Ap_3A hydrolases and their relationship to tumor suppression.

Experimental Section

Enzyme assays: Inhibitory properties of the “supercharged” phosphonates were determined in an incubation mixture (final volume 0.050 mL) containing either 50 mM Mes/KOH, pH 6.9 (for the human Fhit protein, its pH optimum) or 50 mM Hepes/KOH, pH 8.2 (for the lupine Ap_3A hydrolase, its pH optimum), 0.1 mM dithiothreitol, 0.1 mM [^{3}H]-Ap_3A (about 400 000 cpm), 1 mM $MgCl_2$, 1 % of glycerol, 0.1 mg of bovine serum albumin per mL, various concentrations of the inhibitor phosphonate, and a rate-limiting amount of the enzyme. The incubation was carried out at 30 °C. Rates were determined by taking 0.005-mL aliquots at set times (5, 10, 15, and 20 min) and spotting them onto thin layer aluminium plates precoated with silica gel containing fluorescent indicator (Merck). An ADP standard was added, and the chromatogram developed in dioxane/ammonia/water (6/1/4). ADP spots were cut out and the radioactivity counted. The K_i values were calculated from Dixon plots.

  1433-7851/99/3809-1246 $ 17.50+.50/0

Enzyme preparation: Human Fhit protein was overexpressed in *Escherichia coli* as described.[3a] Purification from the crude bacterial extract comprised ammonium sulfate fractionation, ion-exchange chromatography on DEAE-Sephacel, gel filtration on Sephadex G-100, and immobilized-metal-affinity chromatography on TALON (Clonetech) resin from which adsorbed protein was eluted with imidazole (50 mM). Homogeneous dinucleoside triphosphate hydrolase from yellow lupine seeds was obtained as described previously.[22]

Received: November 12, 1998 [Z12656IE]
German version: *Angew. Chem.* **1999**, *111*, 1324 – 1327

Keywords: bioorganic chemistry · enzyme inhibitors · molecular recognition · nucleotides · trisphosphonic acids

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[18] These tripodal analogues have been fully characterized by NMR spectroscopy and mass spectrometry along with all other nucleotide analogues described here. Positive-ion HR-MS: **2**: *m*/*z* 571.971, calcd for $[C_{11}H_{15}N_5O_{12}P_3Na_3+H]^+$: 571.970; **4**: *m*/*z* 1080.898, calcd for $[C_{21}H_{24}ClN_{10}O_{21}P_5Na_6+H]^+$: 1080.896 ($^{35}Cl$); **6**: *m*/*z* 1398.985, calcd for $[C_{31}H_{38}N_{15}O_{27}P_6Na_6+Na]^+$: 1398.977; **8**: 1296.023, calcd for $[C_{31}H_{37}N_{15}O_{24}P_5Na_5+H]^+$: 1296.021. Their purity was established as 98% or greater by HPLC (BioRad MA70 anion exchange column, 50 mM → 700 mM $NH_4^+HCO_3^-$ gradient elution).
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Highly Regio- and Stereoselective Synthesis of Mannose-Containing Oligosaccharides with Acetobromo Sugars as the Donors and Partially Protected Mannose Derivatives as the Acceptors via Sugar Orthoester Intermediates**

Wei Wang and Fanzuo Kong*

Many biologically important natural products such as glycoproteins,[1] the ubiquitous components of the cell membrane, contain an oligomannopyranose core, while the cell wall of yeast contains branched mannans.[2] Mannose-containing oligosaccharides have been synthesized by well-established methods[3] that involve multistep selective protection and deprotection procedures. The use of unprotected or partially protected mannose and acetobromo sugars as raw materials in glycosylations is very attractive for organic chemists because the synthetic routes can be substantially simplified. In previous work[4a] we described a new method for regio- and stereoselective synthesis of oligosaccharides by an orthoester[4d-m] formation/rearrangement procedure with unprotected glucopyranosides as the glycosyl acceptors and acetobromo sugars as the donors which gave 1 →6-linked oligosaccharides in satisfactory yields. In addition, 3-selective glycosylation was achieved with partially protected glucose acceptors containing unprotected 2,3- or 3,4-hydroxy groups. It was found, however, that the glycosylation with unprotected glycosides as the acceptors was rather slow and difficult to monitor owing to the poor solubility of the acceptors in the reaction media. We now report a new strategy for highly regio- and stereoselective synthesis of mannose-containing di- and oligosaccharides via orthoester intermediates by coupling acetobromo sugars with partially protected mannose derivatives as the acceptors, in particular naked mannose 1,2-*O*-ethylidenate.

It is well known that 3,6-branched mannotrisaccharide Manpα1 →6(Manpα1 →3)Man is present in all asparagine-

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[**] This work was supported by the Chinese Academy of Sciences (Project KJ952J$_1$510) and the National Natural Science Foundation of China (Project 29802009).

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