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NOVEL BISPHOSPHONATE INHIBITORS OF PHOSPHOGLYCERATE KINASE

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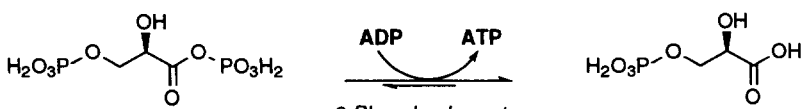
Abstract: A series of novel, conformationally-restrained bisphosphonate analogues of 1,3-*bis*phosphoglyceric acid **1** have been synthesised and evaluated as inhibitors of 3-PGK. They are competitive inhibitors of the human enzyme and, especially for certain α-halophosphonic acid analogues, both K_i and IC_{50} values extend into the submicromolar range.

Introduction 3-Phosphoglycerate kinase (3-PGK, EC 2.7.2.3) is a glycolytic enzyme which converts 1,3-bisphosphoglyceric acid (1,3-BPG) **1** into 3-phosphoglyceric acid and generates ATP.[1] PGK has been regarded as a valid target in the past for the development of agents against liver flukes[2] though there has been little systematic analysis of the features of substrate analogues that should contribute to the development of an inhibitor for PGK.

Scheme 1 Conversion of 1,3-BPG (**1**) into 3-PGA on the glycolytic pathway



1,3-BPG **1** 3-PGA

3-PGK is a bilobal enzyme that has been crystallised from a wide variety of sources.[3-7] The binding site for 3-PGA is found on a 'basic-patch' in the *N*-terminal domain in one lobe[8] and that for the nucleotide is in the *C*-terminal domain in the other lobe.[4] The separation of these two binding sites appears to be too great for in-line phosphoryl transfer and this led Blake[8] to propose a 'hinge-bending' conformational change for the mechanism of action of 3-PGK to bring the reactants together and exclude water. A 'part-closed' structure resulting from such a conformational change has been observed by Wim Hol for the *T. brucei* enzyme in an x-ray structure of 3-PGK with 3-PGA and MgADP where the ternary complex shows a 32° hinge-bending movement relative to the 'open' structure[8] of horse muscle 3-PGK.[9] A similar 'closed' structure for 3-PGK has also been crystallised from *Thermatoga maritima*.[10]

Hitherto, the design of inhibitors for 3-PGK has generally focused on systematic analogues of the natural substrate 1,3-BPG.[11] Our present strategy is to design strong inhibitors for 3-PGK and then seek to modify

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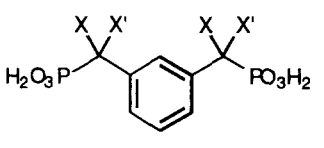
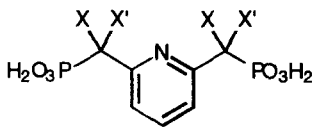
them so as to vary their overall size without significantly weakening enzyme inhibition. In the work described herein, inhibitors of novel design incorporate four main features.[12] Firstly, non-scissile P-C linkages replace the phosphate linkages in 1,3-BPG. Secondly, α-halogenation provides analogues that are isosteric and isopolar with the parent phosphate.[13] Thirdly, an aromatic ring is used as a rigid linker separating the two phosphoryl centres. Fourthly, substituents are introduced on the aromatic ring capable of further elaboration for the generation of bisphosphonates with larger core structures in order both to enhance their binding properties and also to differentiate their inhibition of 3-PGK against that of other enzymes that bind bisphosphates. Structures of these novel bisphosphonates are given below (Figure 1).

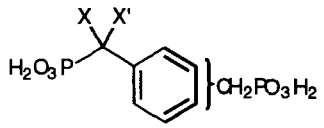
Figure 1 Bisphosphonates synthesised as inhibitors of 3-PGK



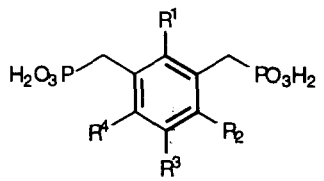
2, X = X' = H;
3, X = H, X' = OH;
4, X = H, X' = Cl;
5, X = H, X' = F;
6, X = X' = F;
7, X = X' = Cl;



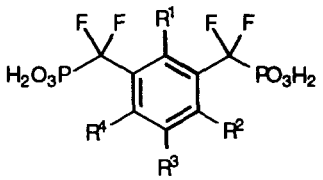
8, X = X' = H;
9, X = H, X' = OH;
10, X = H, X' = Cl;
11, X = H, X' = F;
12, X = X' = F.



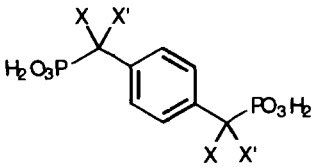
18, X = H, X' = OH;
19, X = H, X' = Cl;
20, X = H, X' = F;
21, X = X' = F.
a = *meta*, **b** = *para*.



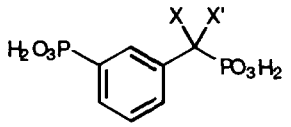
27, R^3 = Me, $R^{1,2,4}$ = H;
28, $R^{2,4}$ = Me, $R^{1,3}$ = H;
29, $R^{1,2,4}$ = Me, R^3 = H;
30, R^1 = F, R^{2-4} = H.



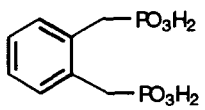
31, R^3 = Me, $R^{1,2,4}$ = H;
32, $R^{2,4}$ = Me, $R^{1,3}$ = H



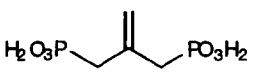
13, X = X' = H;
14, X = H, X' = OH;
15, X = H, X' = F;
16, X = H, X' = Cl;
17, X = X' = F.



22, X = X' = H;
23, X = H, X' = OH;
24, X = H, X' = Cl;
25, X = H, X' = F;
26, X = X' = F.



33



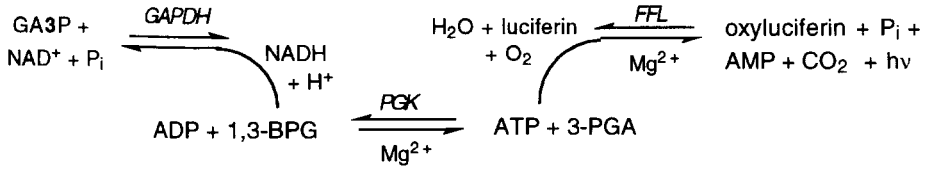
34

All compounds have been fully characterised by spectroscopic and analytical techniques.[12] No separation of stereoisomers has been attempted

Determination of 3-PGK inhibitory activity 3-PGK can be assayed using either the forward or the reverse.reaction (Figure 2). The forward assay system detects ATP formed in a coupled reaction with firefly

luciferase (FFL)[14] whereby the action of FFL on luciferin and ATP yields oxyluciferin and light. The reduction in light emission in the presence of an inhibitor enables determination of IC_{50} values for inhibitors. α-Halogenated bisphosphonates proved strong inhibitors of 3-PGK and were tested at 1 μM concentration while bis-α-hydroxy- and bismethylenephosphonates were used at 10 μM.

Figure 2 Assay of the forward and reverse reactions catalysed by 3-PGK



Kinetic Analysis Kinetics of inhibition of 3-PGK were determined by assay of the reverse reaction[1] in which conversion of 3-PGA into 1,3-BPG is coupled to formation of glyceraldehyde 3-phosphate (GA3P) using GA3P dehydrogenase (GAPDH) (Figure 2), monitored at 340 nm for formation of NADH. A representative group of the above bisphosphonates was selected for kinetic analysis, compounds **2**, **3**, **5**, **8**, **9**, **10**, and **34**. Concentrations of inhibitors at 0.3, 1.0, and 2.5 times IC_{50} were used in kinetic runs for a range of six concentrations of 3-PGA spanning the K_m for 3-PGA (~ 150 μM). The data were graphed as Lineweaver-Burk plots and analysed computationally to identify the nature of the enzyme inhibition and to evaluate K_i (Table 1). In all cases, the quality of fit of the data (not shown) using analysis for competitive inhibition was clearly superior to that for uncompetitive or non-competitive inhibition analysis.

Table 1 K_i values for selected bisphosphonate inhibition of 3-PGK

Compound	K_m[a,b] / μM	K_i[a] / μM	IC_{50}[a,c] / μM
2	71 ± 7.1	120 ± 15	200
3	73 ± 7.5	75 ± 8.5	150
5	74 ± 15	0.69 ± 0.12	1.3
8	77 ± 9	80 ± 10	182
9	95 ± 7	156 ± 17	163
10	84 ± 8	0.33 ± 0.05	1.34
34	75 ± 9	61 ± 7	87

[a] at 37 °C in 0.1 M NaCl, pH 7.1. [b] K_m for 1,3-BPG. [c] IC_{50} values ± 5 %. Results are average of duplicate runs

Determination of pK_a values pK_a values were determined for a selection of 22 of the bisphosphonic acids for the pH range 11 to 3.5. This titration range covers the observed values of pK_a3 and pK_a4 and of the pyridinium NH (in the case of compounds **8**, **9** & **12**). Values were computed using a multiple pK_a analysis programme written for Kaleidagraph™. Experimental pK_a values thus obtained were corrected for statistical factors[15] to take into account the differences in acidity between bisphosphonates having two equivalent phosphonic acid functions and those with non-equivalent phosphonic acids. For non-equivalent bisphosphonic

acids, the assignment of observed pK_a values as pK_a3 and pK_a4 respectively were based on previous results for α-substituted bisphosphonic acids (Table 2).[11,16]

Table 2 IC_{50} values for inhibitors

Compound	IC_{50}[a] / μM	pK_a3, pK_a4	Compound	IC_{50}[a] / μM	pK_a3, pK_a4
2	200	7.11, 7.71	**19a**	8.0	
3	150	6.78, 7.23	**19b**	9.4	
4	1.0		**20a**	8.35	
5	1.3	6.01, 6.37	**20b**	10.7	6.30, 7.72
6	0.96		**21a**	1.8	5.04, 5.84
7	3.6		**21b**	1.98	5.27, 6.63
8	182	6.70, 8.49	**22**	138	6.47, 7.61
9	163	6.57, 7.58	**23**	102	6.55, 7.46
10	1.34		**24**	17	
11	1.33		**25**	16	5.80, 6.99
12	1.17	5.18, 5.30	**26**	1.0	5.16,6.90
13	193	7.03, 7.49	**27**	99	
14	181		**28**	73	7.24, 7.78
15	1.15	6.02, 6.37	**29**	78	7.40, 8.16
16	1.08		**30**	147	
17	0.98	4.82, 5.60	**31**	0.88	
18a	68.8	6.98, 7.29	**32**	0.84	5.33, 5.92
18b	89		**33**	107	7.10, 8.53
			34	87	6.45, 7.45

[a] IC_{50} values are ± 5 %. [b] pK_a values were determined at 37 ± 0.1 °C in 0.152 M NaCl and are accurate to ± 0.05 unit.

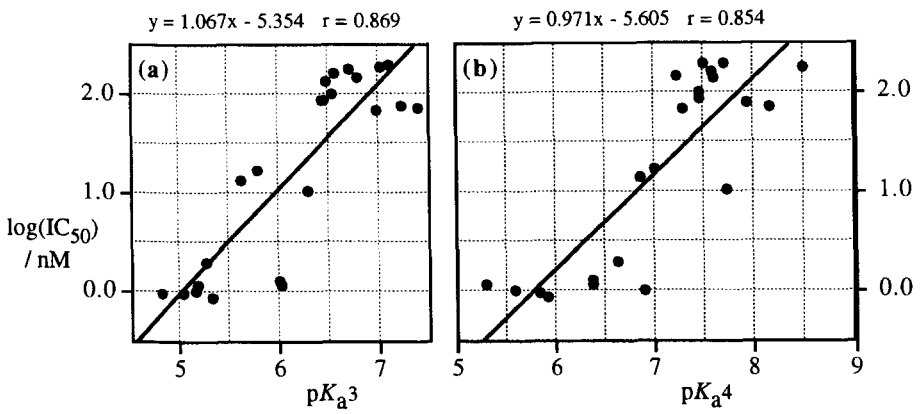
Discussion of results Comparisons between different inhibitors based on their IC_{50} and pK_a values support a number of conclusions about the requirements of the active site of 3-PGK for inhibitor structure.

First, all of the compounds evaluated by K_i determination are *competitive* inhibitors of the enzyme, some showing submicromolar IC_{50} values (Table 1). The values of the inhibition constants, K_i, for the seven compounds determined correlate well with the corresponding values for IC_{50} (Table 1). Both of these values were determined at high sulfate concentration (40 mM) in order to achieve Michaelis-Menten kinetic behaviour[1] for 3-PGK.

Secondly, the rank order of inhibitors is quite uniform through changes in ring-orientation and further phosphonate substitution patterns as $CH_2 < CH(OH) << CCl_2 < CHCl \approx CHF < CF_2$ though the difference is sometimes small for the last three categories. It is clear that α-hydroxylation of the phosphonic acids does not materially enhance their affinity for 3-PGK (Table 2, entries **2** & **3**, **8** & **9**, **13**& **14**, **22** & **23**, *etc.*). By contrast, α-halogenation *always* improves affinity of the bisphosphonic acids for 3-PGK, usually by one to two orders of magnitude and in the best cases leading to submicromolar values for K_i and IC_{50}. This shows the value of isopolar mimicry of the phosphate linkage of the natural substrate while the poorer binding of α-dichloromethylenephosphonates indicates the importance of isosteric quality.[13] In quantitative terms, there is

a modest correlation of IC_{50} with pK_a3 (R = 0.87, Figure 3a) and a similarly modest relationship with pK_a4 (R = 0.85) over the range of 22 bisphosphonates thus evaluated. This analysis appears to suggest that the enzyme responds to the charge on each phosphonate group independently. What is most clearly shown by these data is that to achieve high affinity for 3-PGK, the inhibitors must have a pK_a3 value below 6.5 (Figure 3a) and also a pK_a4 below 7 (*cf.* **20b**, **22**, **23**, **25**) (Figure 3b).

Figure 3 $\log(IC_{50})$ *vs* pK_a for Bisphosphonate Inhibitors of 3-PGK



Thirdly, 3-PGK appears to tolerate bisphosphonates with additional steric components (e.g. **27** - **32**) where in some cases strain must force the two phosphoryl functions to lie outside the plane of the benzene ring (notably **29**). Also, 3-PGK shows little discrimination against compounds with a separation of phosphoryl centres greater than that of the natural substrate, notably in the series of *p*-substituted unsymmetrical *p*-xylylbisphosphonates (**18b**-**21b**). This kinase can, however, show good binding to inhibitors with fewer atoms linking these phosphoryl centres (**24**-**26**) though there appears to be a lower limit to this tolerance (**34**).

Lastly, the unsymmetrical benzenephosphonic acid inhibitors with one α-halo- and especially one α,α-difluoromethylenephosphonate substituent show activity at least as good as their bismethylenephosphonate counterparts with two such centres. This observation is borne out by comparison of the pairs **4** - **19a**, **5** - **20a**, and **6** - **21a**. Further studies will be necessary to determine whether these unsymmetrical inhibitors bind in one particular orientation to the basic patch of 3-PGK, though such behaviour has been detected in work on some unsymmetrical azapentane-1,5-bisphosphonates[11,17] and may thereby differentiate between phosphonate mimicry of the transferable phosphate in 1,3-BPG and the inert one at C-3.

In conclusion, the present results provide one of the clearest cut examples yet observed of the value of α-fluorination of phosphonic acids to improve their affinity for proteins as non-hydrolysable enzyme inhibitors or substrate analogues.

Experimental Part-purified human 3-PGK was isolated by a modification of the procedure of Yoshida.[18] The isolated enzyme was assayed spectrophotometrically[18] for the back reaction at high 3-PGA concentration and found to have an activity of 12.5 u/ml. 1,3-BPG was prepared according to the method of Negelein[19] and ADP (ATP-free) according to the method of Pogson *et al.*[20] All other compounds were purchased from Sigma

and used as received. Dithiothreitol was added at 5 μM concentration to stabilised GAPDH. The forward assay was performed according to the published procedure.[15,20] Lineweaver-Burk plots were determined using Origin™ and this programme was also used to calculate K_i values and error factors for the kinetic data. pH titration profiles were plotted from the titration data using software written for Kaleidagraph™.

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