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**Abstract:** A series of novel, conformationally-restrained bisphosphonate analogues of 1,3-bisphosphoglyceric acid 1 have been synthesised and evaluated as inhibitors of 3-PGK. They are competitive inhibitors of the human enzyme and, especially for certain  $\alpha$ -halophosphonic acid analogues, both  $K_i$  and  $IC_{50}$  values extend into the submicromolar range. © 1998 Elsevier Science Ltd. All rights reserved.

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

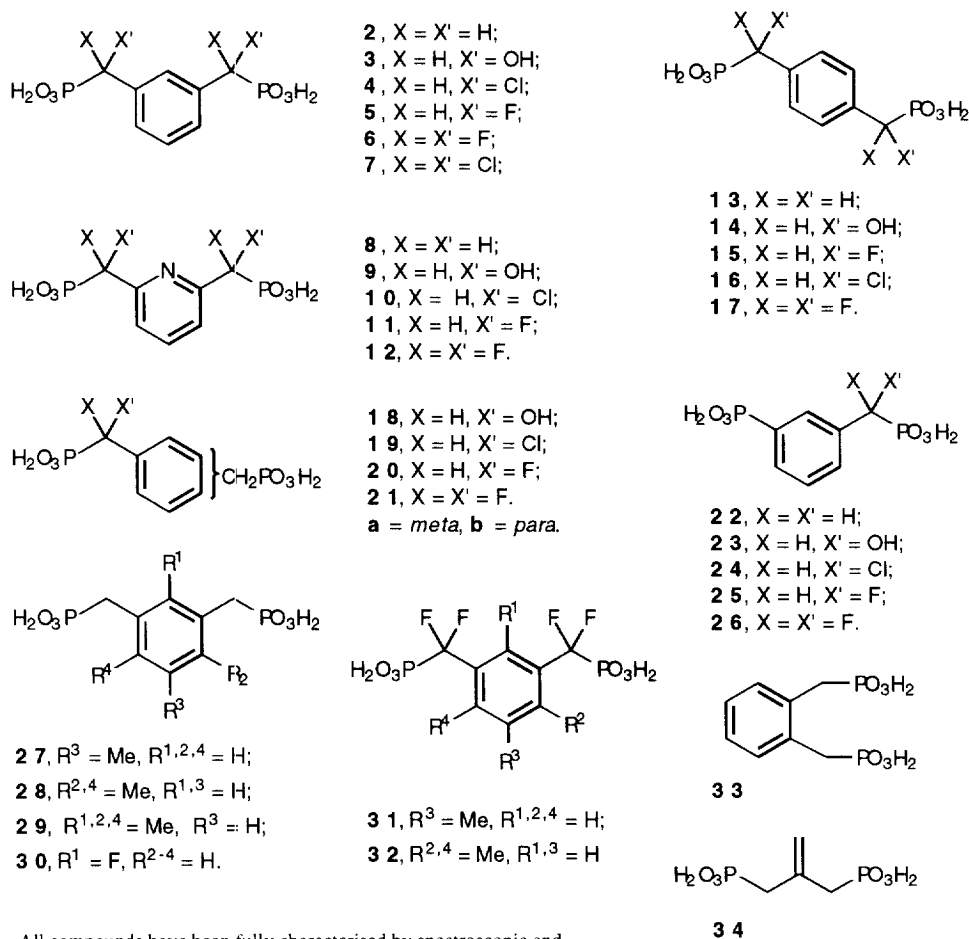


Hitherto, the design of inhibitors for 3-PGK has generally focused on systematic analogues of the natural substrate 1,3-BPG.<sup>11</sup> Our present strategy is to design strong inhibitors for 3-PGK and then seek to modify

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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.<sup>12</sup> Firstly, non-scissile P-C linkages replace the phosphate linkages in 1,3-BPG. Secondly,  $\alpha$ -halogenation provides analogues that are isosteric and isopolar with the parent phosphate.<sup>13</sup> 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

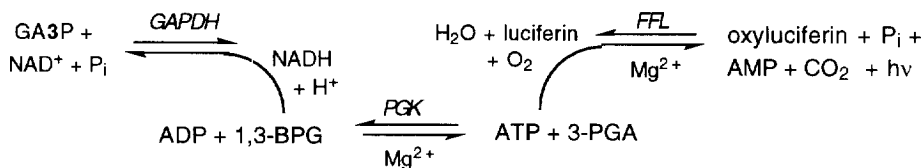


All compounds have been fully characterised by spectroscopic and analytical techniques.<sup>12</sup> No separation of stereoisomers has been attempted

**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)<sup>14</sup> 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<sub>50</sub> values for inhibitors.  $\alpha$ -Halogenated bisphosphonates proved strong inhibitors of 3-PGK and were tested at 1  $\mu$ M concentration while bis- $\alpha$ -hydroxy- and bismethylenephosphonates were used at 10  $\mu$ M.

**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<sup>1</sup> 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<sub>50</sub> were used in kinetic runs for a range of six concentrations of 3-PGA spanning the  $K_m$  for 3-PGA ( $\sim 150 \mu\text{M}$ ). 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	$K_m^{a,b} / \mu\text{M}$	$K_i^a / \mu\text{M}$	$\text{IC}_{50}^{a,c} / \mu\text{M}$
<b>2</b>	$71 \pm 7.1$	$120 \pm 15$	200
<b>3</b>	$73 \pm 7.5$	$75 \pm 8.5$	150
<b>5</b>	$74 \pm 15$	$0.69 \pm 0.12$	1.3
<b>8</b>	$77 \pm 9$	$80 \pm 10$	182
<b>9</b>	$95 \pm 7$	$156 \pm 17$	163
<b>10</b>	$84 \pm 8$	$0.33 \pm 0.05$	1.34
<b>34</b>	$75 \pm 9$	$61 \pm 7$	87

<sup>a</sup> at 37 °C in 0.1 M NaCl, pH 7.1 <sup>b</sup>  $K_m$  for 1,3-BPG. <sup>c</sup> IC<sub>50</sub> values  $\pm 5\%$ . 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<sup>15</sup> 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  $\alpha$ -substituted bisphosphonic acids (Table 2).<sup>11,16</sup>

**Table 2**  $IC_{50}$  values for inhibitors

Compound	$IC_{50}^a / \mu M$	$pK_{a3}, pK_{a4}$	Compound	$IC_{50}^a / \mu M$	$pK_{a3}, pK_{a4}$
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

<sup>a</sup>  $IC_{50}$  values are  $\pm 5\%$ . <sup>b</sup>  $pK_a$  values were determined at  $37 \pm 0.1^\circ C$  in 0.152 M NaCl and are accurate to  $\pm 0.05$  unit.

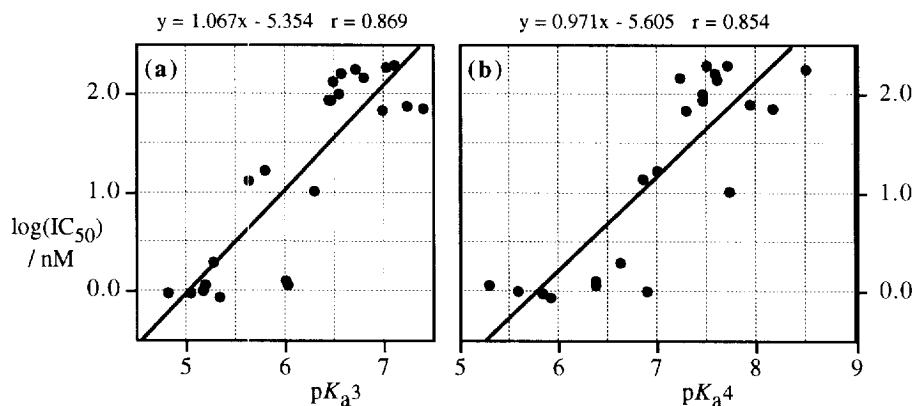
**Discussion of results** Comparisons between different inhibitors based on their  $IC_{50}$  and  $pK_a$  values support a number of conclusions about the requirements of the active site of 3-PGK for inhibitor structure.

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<sup>1</sup> for 3-PGK.

Secondly, the rank order of inhibitors is quite uniform through changes in ring-orientation and further phosphonate substitution patterns as  $CH_2 < CH(OH) \ll CCl_2 < CHCl \approx CHF < CF_2$  though the difference is sometimes small for the last three categories. It is clear that  $\alpha$ -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,  $\alpha$ -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  $\alpha$ -dichloromethylenephosphonates indicates the importance of isosteric quality.<sup>13</sup> 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  $\alpha$ -halo- and especially one  $\alpha,\alpha$ -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<sup>11,17</sup> 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  $\alpha$ -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.<sup>18</sup> The isolated enzyme was assayed spectrophotometrically<sup>18</sup> 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<sup>19</sup> and ADP (ATP-free) according to the method of Pogson *et al.*<sup>20</sup> All other compounds were purchased from Sigma

and used as received. Dithiothreitol was added at 5  $\mu$ M concentration to stabilised GAPDH. The forward assay was performed according to the published procedure.<sup>15,20</sup> 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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