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SYNTHESIS OF PHOSPHONATE ANALOGUES OF 1,3-*BIS*PHOSPHOGLYCERIC ACID AND THEIR BINDING TO YEAST PHOSPHOGLYCERATE KINASE

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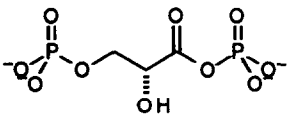
Abstract: Phosphonate analogues of 1,3-*bis*phosphoglyceric acid, several incorporating non-scissile α-difluoro phosphonates, have been synthesised and their binding to phosphoglycerate kinase measured by NMR analysis. The tetrafluoroamide analogue (**4**) of 1,3-P_2GA shows the strongest binding, with a dissociation constant of 2 μM.

Introduction

3-Phosphoglycerate kinase, PGK (EC 2.7.2.3), is a key enzyme in the glycolytic pathway. It generates ATP by the equilibration of phosphate transfer between position-1 of 1,3-*bis*phosphoglyceric acid (1,3-P_2GA, **1**) and the γ-position of adenosine triphosphate (ATP, Eq. 1).



(1)

$$1,3\text{-}P_2GA + ADP \underset{}{\overset{Mg^{2+}}{\rightleftharpoons}} 3\text{-}PGA + ATP \quad -4.8\ kcalmol^{-1} \qquad \textbf{(Eq.1)}$$

The enzyme has been crystallised from a variety of sources[1-4] It has a bilobal structure, one substrate binding in each lobe. The distance between the substrates once bound to the 'open' form of the enzyme is 12-15Å, which is too great for direct phosphoryl transfer without a major conformational change throughout the enzyme. It has been proposed that PGK undergoes a 'hinge bending' motion[1, 5] which brings the two lobes together to allow phosphoryl transfer. This 'closed' state of the enzyme has been probed by various studies. These include low angle X-ray scattering,[6, 7] sedimentation velocity measurements, 1D and 2D NMR experiments[8] together with the use of paramagnetic β,γ-bidentate CrATP[9] or RhATP[10] analogues of MgATP, and X-ray crystallography.[11] However, the catalytically active, 'closed' form of yeast PGK has not yet been identified in molecular detail.

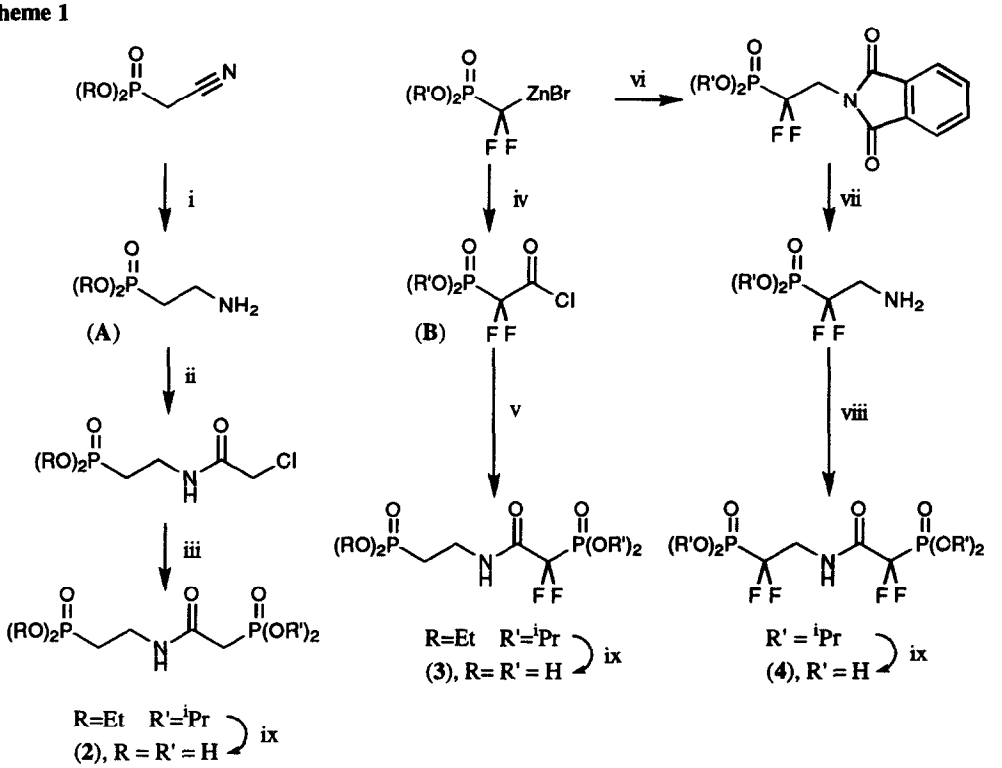
The inhibition of PGK has been effected by large anionic molecules such as the trypanocidal drug Suramin,[12] which is an effective inhibitor of PGK from *Trypanosoma brucei*[13] but less potent for yeast PGK. Previous molecules designed to mimic either 1,3-P_2GA or ATP have generally failed to inhibit yeast PGK.[14] Recently some keto-bisphosphonates have been investigated[15] as a new class of reversible inhibitors for the enzyme. These smaller anionic compounds probably act as analogues of 1,3-P_2GA which bind at the 1,3-P_2GA binding site, which has been defined[2] as a basic patch on the N-terminal domain of the kinase.

We have applied rational drug design to produce a series of carbonyl-containing analogues (**2-4**) of 1,3-P_2GA (**1**) to bind to PGK, together with fluorinated and non-fluorinated alkyl and ether *bis*phosphonates (**5-10**). The CF_2 moiety has been incorporated into these analogues as a non-scissile, isosteric and isoelectronic replacement for an oxygen atom which improves the physicochemical parameters associated with a phosphonate, especially the state of ionisation of a phosphonic acid.[16] The dissociation constants (K_d) of these compounds have been measured by NMR titration experiments.

Analogue design and synthesis

In earlier preliminary studies (W. Fairbrother, D.Phil Thesis, Oxford University, 1989) pentane-1,5-bisphosphonic acid (**6**) had been found to be a rather poor inhibitor for PGK. We therefore decided that a successful analogue of 1,3-P_2GA should have three features. Firstly, there should be maximum negative charge separated by a chain of five atoms, as in the parent species (**1**). Secondly, that chain should not contain any scissile P-O bond. Thirdly, there should be a carbonyl group at position-2 in the chain as a potential H-bond acceptor and an H-bond donor at position-3.

Scheme 1

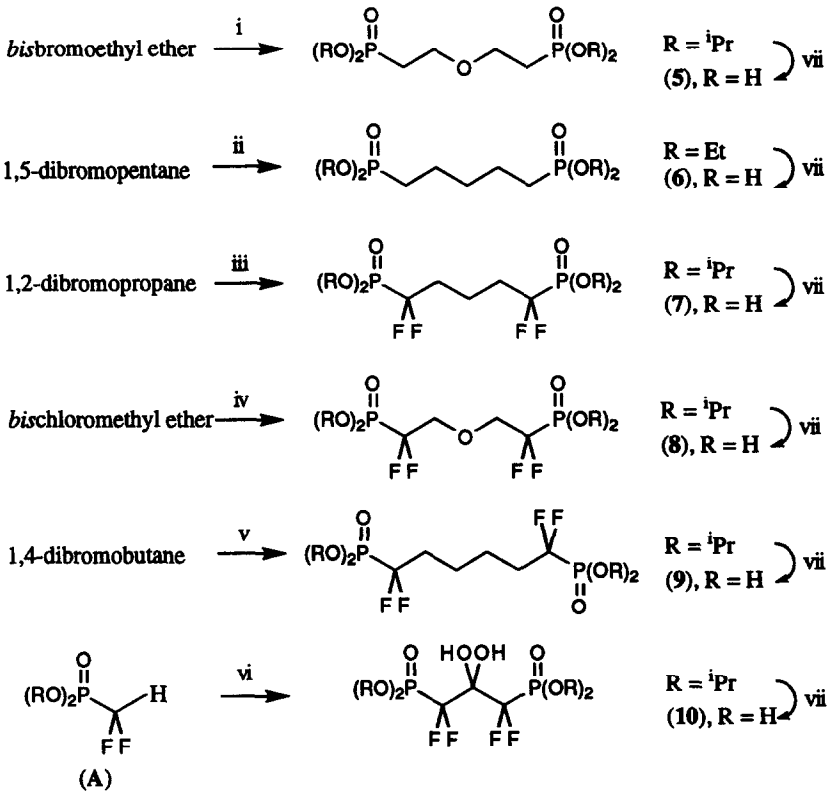


(i) H_2, $PtO_2.xH_2O$, MeOH, 98%; (ii) $(ClCH_2CO)_2O$, NEt_3, DCM, 40%; (iii) $P(OR')_3$, Δ, 77%
(iv) (a) Cu(I)Br,[17] ClCOOEt, monoglyme, (b) KOH, ethanol, 80%, (c) $(COCl)_2$; (v) (**A**), NEt_3, DCM, 50%; (vi) Cu(I)Br,[17] *N*-(Bromomethyl)phthalimide, monoglyme, 30%; (vii) $NH_2NH_2.H_2O$, DCM, 70%; (viii) (**B**), NEt_3, DCM, 89%; (ix) TMSBr, DCM, (**2**) 89%; (**3**) 95%; (**4**) 40%

Our previous work on the isoelectronic advantages of α-fluorophosphonates[16] encouraged us to include such functionality at either or both positions in the linker chain, as in compounds (**7** and **9**), and also a minimum lone-pair donor, as in compound (**8**). However, the introduction of a C-2 carbonyl group into such species would result either in its existence in an enolic form or generate a very labile difluoroacetate ester. We therefore elected to work on the amide analogues (**2-4**), which provide an H-bond donor at position-3 of the spacer chain while circumventing the problem of chirality on the 2-hydroxy group of (*R*)-glyceric acid . Our attempts to improve the mimicry further by introducing a hydroxyl substituent at position-3, *via* *N*-hydroxyamides, led to compounds insufficiently stable for use.[18]

Chemical synthesis of the *bis*phosphonates proceeded according to Scheme 1 for compounds (**2-4**). The alkyl and ether *bis*phosphonates (**5-10**) were synthesised by the routes shown in Scheme 2. The compounds were deesterified using TMSBr or HCl and then titrated to pH 7.1 with NaOH for subsequent enzyme binding studies.

Scheme 2



(i) $P(OR)_3$, 86%; (ii) $NaPO(OR)_2$, 65%; (iii) (**A**), LDA, THF, -78°C, 46%; (iv) (**A**), LDA, THF, -78°C, 16%; (v) (**A**), LDA, THF, -78°C, 17% (isolated as a side product); (vi) LDA, THF, -78°C, CO_2, 60%; (vii) HCl (6M), (**5**) 85%; (**6**) 96%; (**7**) 73%; (**8**) 97%; (**9**) 91%; (**10**) 95%

Biological activity

NMR studies on phosphoglycerate kinase using wild-type and mutant enzyme have led to the partial assignment of the enzyme, in particular of His62, His167 and His170 (yeast numbering). These residues are within the basic patch of the enzyme and their chemical shifts change when substrates interact with PGK at the 1,3-P_2GA site. The chemical shift of each of these residues was monitored as a function of the concentration of added *bis*phosphonic acid. Spectra were recorded on a Bruker AMX 500 spectrometer at 300 K, pH 7.1 in triethanolamine buffer (10 mM) and KCl (40 mM), with acetone as internal reference. The concentration of yeast PGK (Boehringer) was measured by UV absorbance at 280 nm.

Aliquots of analogue solution (1-2 μl, 10 mM) were added to PGK (0.3 mM) in D_2O. The change in chemical shift of His62 on titration of PGK with three of the analogues tested is shown in Figure 1. Independent estimates of K_d were obtained from fitting theoretical dissociation curves to the shifts of His62, His167 and His170 and the dissociation equilibrium constant, K_d, calculated by least squares analysis. The results are summarised in Table 1, in which the R_{factor} values provide a measure of the goodness of fit. The values derived from each of the three histidine residues are in good accord. Data in Table 1 are also provided for K_d for the substrate, 3-phosphoglyceric acid.

Table 1 Equilibrium binding constants of 1,3-P_2GA analogues with yeast Phosphoglycerate Kinase

Compound	K_d / μM and (R_{factor}) / %		
	His62	His167	His170
3-Phosphoglyceric acid	112 (8)	94 (6)	121[a] (12)
(**2**)	420 (6)	543 (10)	481 (14)
(**3**)	8 (14)	4 (16)	7 (28)
(**4**)	2 (23)	4 (23)	7 (28)
(**5**)	[b]	5882 (23)	4115 (20)
(**6**)	1300 (20)	[b]	[b]
(**7**)	303[a] (34)	119 (8)	160 (10)
(**8**)	126 (5)	253 (7)	331 (9)
(**9**)	920[a] (6)	1320 (6)	680[a] (12)
(**10**)	80 (7)	71 (13)	66 (10)
SO_4^{2-}	1410 (7)	1650 (8)	775 (8)

[a] Chemical shift upfield rather that the usual downfield. [b] Data too inconsistent for meaningful K_d.

Discussion

Pentane-1,5-bisphosphonic acid (**6**), the most simple analogue of 1,3-P_2GA, is a very weak binder to yeast PGK with an affinity even lower than that of sulphate. Indeed, the effects on His167 and His170 were so weak as to make their analysis impracticable. The introduction of an ether oxygen at position-3 with 3-oxa-pentane-1,5-bisphosphonic acid (**5**) led to a decrease in binding to the enzyme. By contrast, α-fluorination led to an enhancement of binding for both the 5-carbon alkane (**7**), its homologue (**9**), and the related ether (**8**). It is clear that a 5-atom spacer is superior to a 6-atom spacer between the two phosphonates and that α-fluorination very

significantly potentiates binding to PGK, presumable as a result of the increased negative charge in (**7**) relative to (**6**) and (**8**) relative to (**5**). Rather surprisingly, the gem-diol (**10**) is a very effective binder notwithstanding the attenuation of the length of its spacer chain to three atoms.

Our initial studies of the non-fluorinated amide (**2**) provided encouraging results, with nicely consistent data emerging from the analysis of all three histidines. The major binding improvements over (**5**) and (**6**) establish the importance of the carbonyl and H-bond donor elements in the spacer chain. These are very strongly enhanced by α-fluorination, as in compounds (**3**) and (**4**). While one cannot ignore the potential hydrophobic effects of α-fluorination on these phosphonate analogues of the parent phosphate esters, it would appear that the increase in charge density resulting from such fluorination is more important in the binding locus for the 1-phosphate of 1,3-P_2GA, as evidenced by a 100-fold reduction in K_d for (**3**) relative to (**2**), than for the 3-phosphate, where α-fluorination produces only a small additional increase in binding for (**4**) relative to (**3**).[19] What is abundantly clear is that α-fluorination and the amide functionalities generate commutative effects with respect to binding in the basic patch of yeast PGK that, taken together, provide the first, analogues of 1,3-P_2GA with micromolar binding affinities.

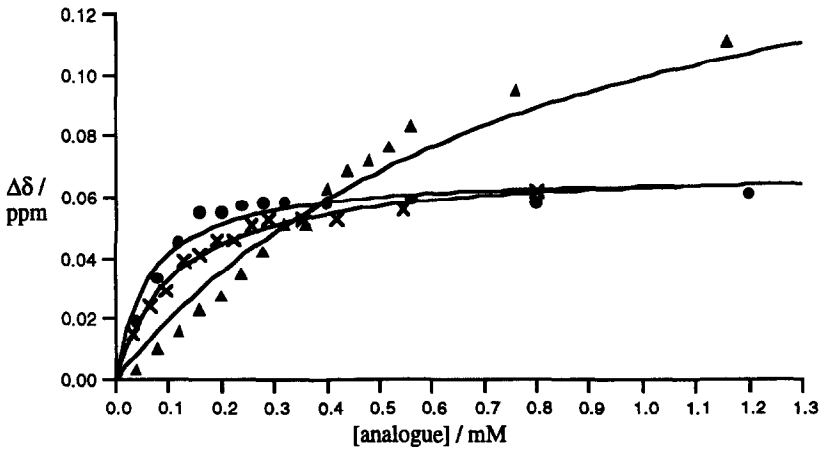


Figure 1 The change in chemical shift of His62 on PGK titration with analogues (**2**) (▲), (**3**) (X), and (**4**) (●) as a function of their concentration. Continuous lines represent theoretical dissociation curves for a one-to-one complex.

Conclusion

The α–fluorination of bisphosphonates separated by a 5-atom spacer provides an excellent series of analogues of 1,3-P_2GA as tight binders to yeast PGK. Their potency is further enhanced by the introduction of an amide functionality to mimic the 2-hydroxycarboxylate functionality of (*R*)-glyceric acid.

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