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Selective Inhibition of *Trypanosoma Brucei* GAPDH by 1,3-Bisphospho-D-glyceric Acid (1,3-diPG) Analogues

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Abstract—Various phosphono-phosphates and diphosphonates were synthesized as 1,3-diphosphoglycerate (1,3-diPG) analogues by using a β-ketophosphonate, an α-fluoro,β-ketophosphonate or a β-ketophosphoramidate to mimic the unstable carboxyphosphate part of the natural substrate. The inhibitory effect of these analogues on glyceraldehyde-3-phosphate dehydrogenases (GAPDH) from *Trypanosoma brucei* (Tb) and rabbit muscle were measured with respect to both substrates, glyceraldehyde-3-phosphate (GAP) and 1,3-diPG. Interestingly, all 1,5-diphosphono,2-oxopentanes without substitution at the C-3 position selectively inhibit the Tb GAPDH with respect to 1,3-diPG and are without effect on Rm GAPDH. All 1-phospho,3-oxo,4-phosphonobutanes show themselves to be non-selective inhibitors either with regard to substrates or organisms, but they will be of a great interest as 1,3-diPG stable models for structural studies of co-crystals with GAPDHs. © 2001 Elsevier Science Ltd. All rights reserved.

Introduction

Trypanosomes are flagellated protozoa responsible for serious parasitic diseases in humans (sleeping sickness), domestic animals, fish and plants in subtropical and temperate regions. Today, the trypanosomiases represent a formidable medicinal and economic obstacle to development in African and South American countries and rank among the first tropical diseases selected by the World Health Organization to develop new or more effective treatments.[1] Owing to toxicity and lack of efficacy, most of the chemotherapies currently used remain unsatisfactory and the design of novel classes of anti-trypanosomatid drugs has become urgent.

Opperdoes et al.[2,3] have shown that, due to the obligatory dependence of the bloodstream form of *Trypanosoma brucei* on glycolysis for ATP production, the glycolytic enzymes are good targets for drug design. The glycosomal glyceraldehyde-3-phosphate-dehydrogenase (GAPDH)[4,5] has previously been considered as an important target for the design of trypanocidal drugs.[6–8] This enzyme reversibly catalyses the oxidative phosphorylation of D-glyceraldehyde-3-phosphate (GAP) into 1,3-diphospho-D-glyceric acid (1,3-diPG) in the presence of NAD^+ and inorganic phosphate (Scheme 1). The most potent and selective inhibitors of the GAPDH from parasites described to date are adenosine analogues.[7] However, as the GAPDH natural substrate 1,3-diPG is able to cross the glycosomal membrane,[9] selective inhibitor analogues of this substrate will probably offer the advantage of an easy uptake by this parasite's microbody. Their first uptake into cytosol across the plasma membrane should probably become possible from lipophilic prodrug phosphate analogues.[10]

In order to design selective and specific inhibitors for *T. brucei* GAPDH, we developed two families of 1,3-diPG substrate analogues: 1,5-diphosphonate-2-oxopentane and 1,4-phosphonophosphate-2-oxobutane (Schemes 2–5).

In a first step, inhibitor structures were selected to provide the closest homology with the substrate 1,3-diphosphoglycerate, keeping the overall size, the diphosphoryl moieties, the carbonyl at the C-3 position and the (*R*) configuration for carbon C-2 bearing the hydroxyl group. Indeed, owing to the low stability of the mixed anhydride part in 1,3-diPG ($t_{1/2} = 30$ s),[11] this moiety was replaced by a β-ketophosphonate structure.

In a second step, from results obtained in inhibition studies of GAPDHs with these compounds, all structural modifications were carried out on the β-ketophosphonate moiety to lead to a set of selective inhibitors having

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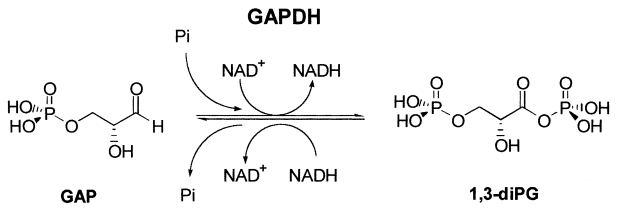
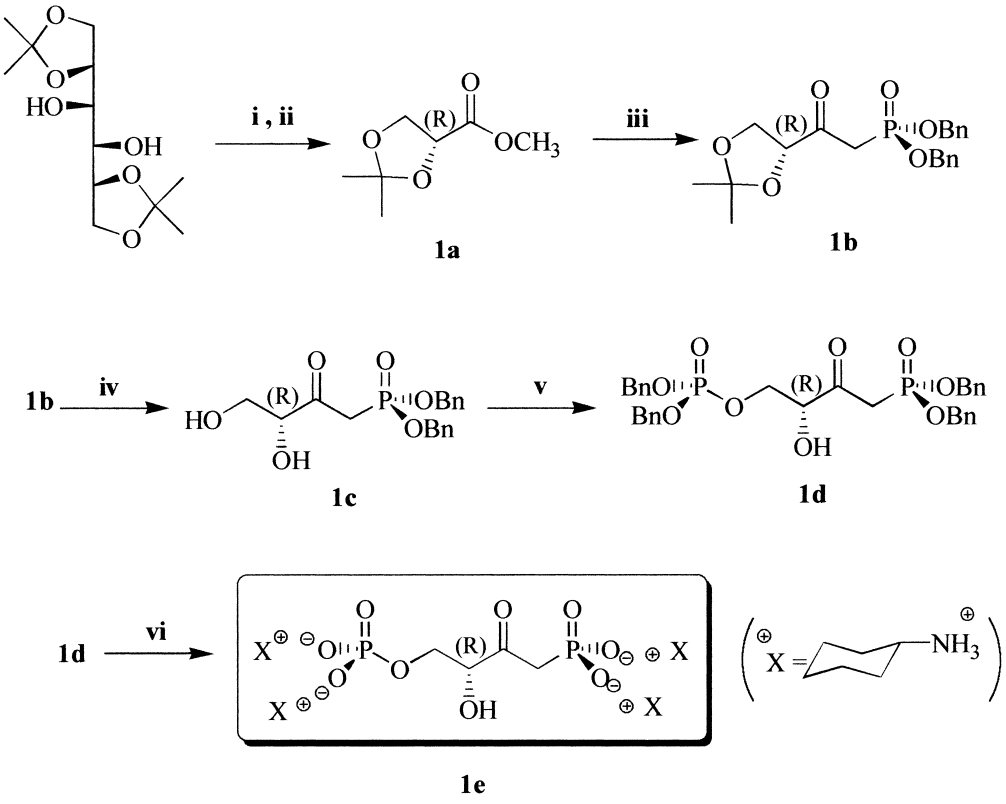


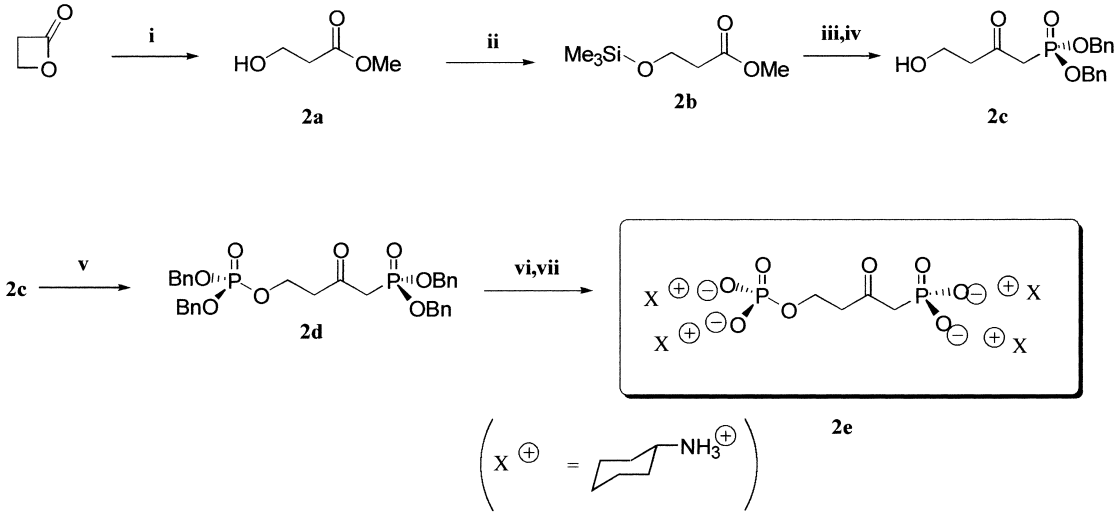
Scheme 1. Reversible reaction catalyzed by GAPDH.

improved affinities for the enzyme from *T. brucei*. We report here their synthesis and evaluation as GAPDH inhibitors from *Trypanosoma brucei* and from rabbit muscle (chosen as the mammalian model).

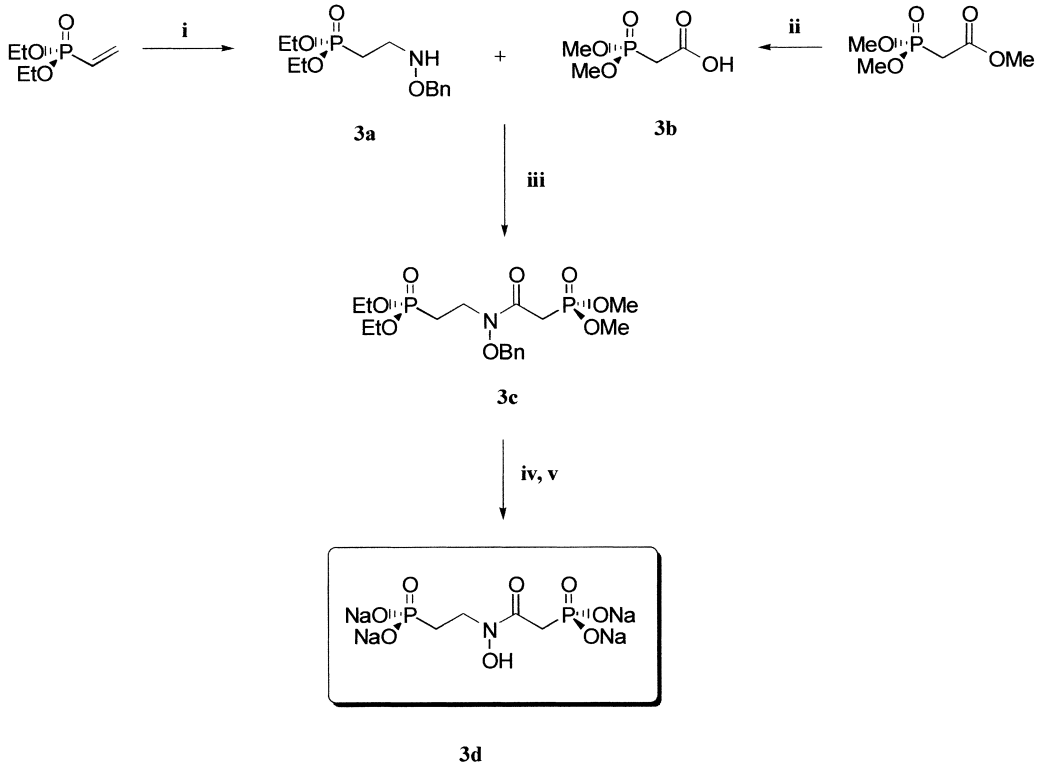
We will distinguish (i) non-selective inhibitors (such as the original phosphono-phosphate **1e** (Scheme 2), 1,3-diPG isosteric analogue) that may, by co-crystallization with GAPDH, provide valuable information on these



Scheme. 2. Reagents and conditions: (i) $NaIO_4$, H_2O, CH_2Cl_2, rt (95%); (ii) Br_2, $NaHCO_3$, MeOH/H_2O (9/1), rt (80%); (iii) $CH_3P(O)(OBn)_2$, BuLi, THF, −80 °C (90%); (iv) HCl 1 N, THF/H_2O, rt (40%); (v) $P(OBn)_3$, I_2, pyridine, CH_2Cl_2, −30 °C (50%); (vi) Pd/C, H_2, MeOH, cyclohexylamine, rt (100%).



Scheme 3. Reagents and conditions: (i) NaOH, MeOH (80%); (ii) Me_3SiCl, pyridine, CH_2Cl_2 (90%); (iii) $(BnO)_2P(O)CH_3$, BuLi, THF, −80 °C; (iv) HCl, pH = 1 (75%); (v) $P(OBn)_3$, I_2, pyridine, CH_2Cl_2, 0 °C (75%); (vi) H_2, Pd/C, MeOH (100%); (vii) cyclohexylamine.



Scheme 4. Reagents and conditions: (i) NH_2OBn, EtOH, 60 °C, 4 days (55%); (ii) NaOH, H_2O (98%); (iii) *N*-(3-dimethylaminopropyl)-*N'*-ethyl-carbodiimide hydrochloride, THF/H_2O, pH = 5 (86%); (iv) H_2, Pd/C, MeOH (98%); (v) Me_3SiBr, NaOH (97%).

substrate's binding sites, and (ii) the first 1,5-diphosphonate-2-ketopentanes which selectively inhibit parasite GAPDH by competition with the natural substrate 1,3-diPG.

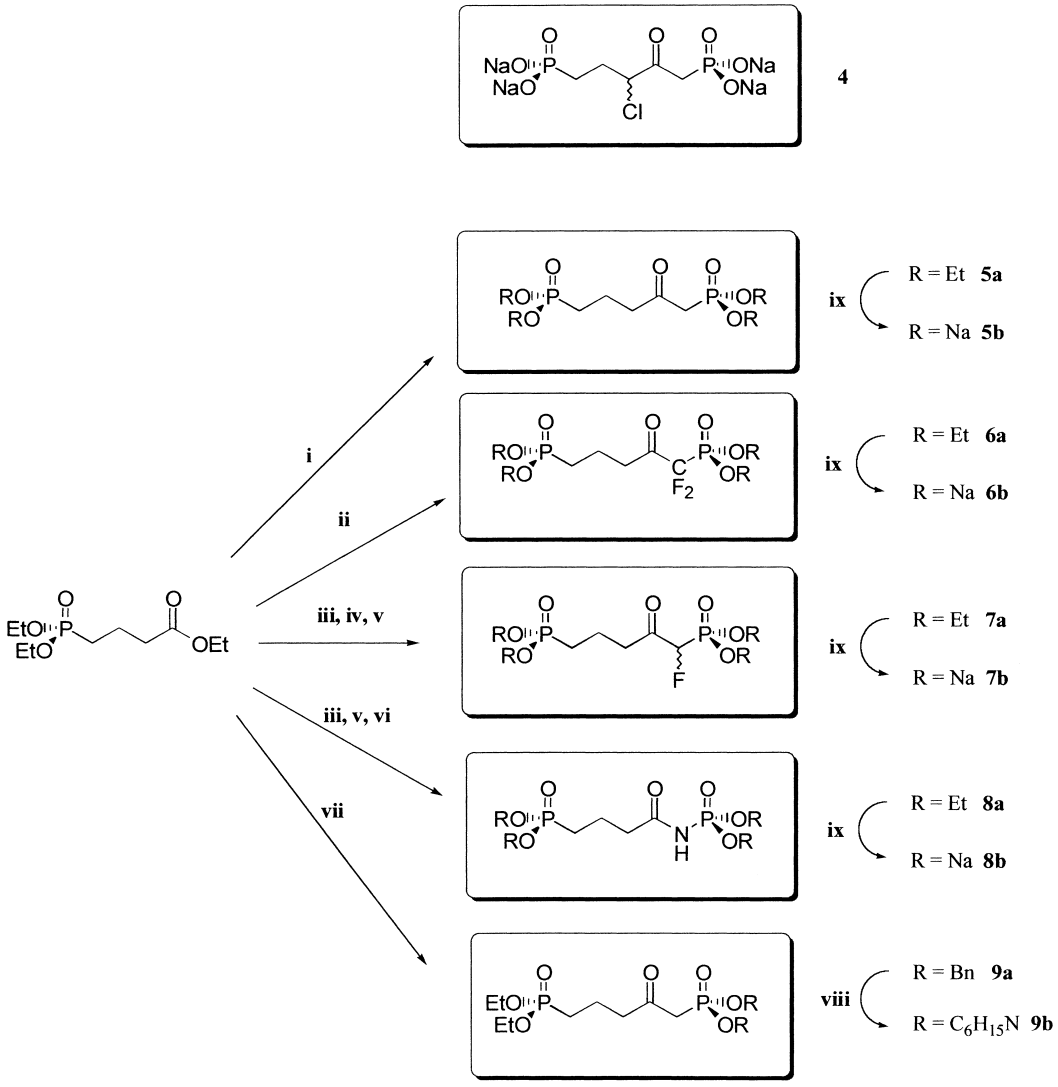
Results and Discussion

Phosphono-phosphates **1e** and **2e** (Schemes 2 and 3) have as precursors *R*-methylglycerate and methyl-4-hydroxypropanoate, respectively. Phosphonate and phosphate groups were successively introduced on the triose frame using dibenzylmethylphosphonate, and subsequently tribenzylphosphite with iodine. These phosphorylating agents were prepared by previously described synthetic procedures.[12,13]

Concerning compound **1** (Scheme 2), oxidative cleavage of 1,2,5,6,-di-*O*-isopropylidene-D-mannitol by sodium periodate gave two equivalents of 2,3-di-*O*-isopropylidene-D-glyceraldehyde[14] with an *R* configuration at the C-2 position, as in the GAPDH natural substrate. The unstable aldehyde was immediately transformed into a carboxylate group by oxidative reaction of the couple Br_2/H_2O, followed by an in situ esterification with methanol in neutral conditions. Compound **1a** was firstly phosphorylated by condensation of the dibenzylmethylphosphonate carbanion on the methyl ester. Deprotection of the diol by acidic hydrolysis of the isopropylidene bridge without hydrolyzing the di-*O*-benzyl groups was carried out in successive steps: the reaction, followed by ^{31}P NMR analysis, was stopped as soon as the concentration of monobenzylphosphonate increased to approximately 20% and the products were then separated. The next step was the monophosphorylation of diol **1c**. This was done without protection of the secondary alcohol group, using the tribenzylphosphite-iodine couple as reagent.

Compound **2e** (Scheme 3) is derived from methyl-3-hydroxypropanoate resulting from the base-catalysed ring-opening of β-propiolactone that proceeds mainly by nucleophilic attack of methanol on the carbonyl group.[15] After a trimethylsilyl protection of the primary hydroxyl group, the phosphonylation was obtained by nucleophilic attack on the ester group of a carbanion species, as described for **1b**. The phosphorylation of **2c** was performed using the same procedure as for compound **1d**, namely $P(OBn)_3/I_2$. As the obtained phosphono-phosphate **2d** was very sensitive to acidic conditions (purification on silica gel led, by phosphate elimination, to 3,4-ene-2-oxobutanephosphonate), the purification was performed by HPLC on a C_{18} silica gel column. Deprotection, in neutral conditions, of the four benzyl groups by catalytic hydrogenation led to **2e** as a tetracyclohexylammonium salt.

In order to maintain an OH group at the C-2 position as in the natural substrate, the hydroxylamide **3d** was synthesized (Scheme 4). It was obtained by formation of an amidic bond between dimethyl phosphono-acetic acid **3b** and diethylphosphonoethyl-(*O*-benzyl)amidoxime. The phosphorylated intermediate **3a**, arose from an addition reaction of benzylhydroxylamine on diethylvinylphosphonate.[16] Total deprotection of the different hydroxyl groups was successively carried out in two steps by (i)



Scheme 5. Reagents and conditions: (i) $(EtO)_2P(O)CH_3$, BuLi, THF, −80 °C (77%); (ii) $(EtO)_2P(O)CF_2H$, LDA, THF, −80 °C (72%); (iii) NaOH (95%); (iv) $SOCl_2$, CH_2Cl_2 (100%); (v) $(EtO)_2P(O)CHFCOOH$, BuLi, THF, −80 °C (20%); (vi) $(EtO)_2P(O)NH_2$, Et_3N, CH_2Cl_2 (25%); (vii) $(BnO)_2P(O)CH_3$, BuLi, THF, −80 °C (85%); (viii) H_2, Pd/C, MeOH, cyclohexylamine (98%); (ix) Me_3SiBr, NaOH (95–100%).

catalytic hydrogenation of *N-O*-benzyl, and then (ii) acidic hydrolysis of the phosphoric tetrasilylester intermediates resulting from the reaction with trimethylbromosilane.[17]

For the synthesis of other compounds, the β-ketophosphonate moiety was modified by introducing fluorine or nitrogen atoms to restore, to the maximum possible, the pK and geometry of the mixed anhydride (O)C–O–P(O) moiety of 1,3-diPG.

The diphosphonate analogues **5**–**9** (Scheme 5) were synthesized from commercially available triethyl-4-phosphonobutyrate by different coupling reactions depending on the nature of the substituent at carbon C-1. Compounds **5** and **6** were synthesized as previously described[18] by nucleophilic attack of the ester group by the carbanion obtained from diethylmethyl (or difluoromethyl) phosphonate. For compound **9**, the dibenzylmethylphosphonate carbanion was condensed on triethyl-4-phosphonobutyrate leading to a diphosphonate with differently protected phosphoryl groups. Catalytic hydrogenation allowed the selective deprotection of the benzyl groups borne by the phosphonate group at carbon 1; the affinity of this compound **9b** will be compared to that of compound **5b**. This will provide valuable information on the importance of each phosphonate group on the inhibitory effects.

Finally, α-monofluoro-β-ketophosphonate **7b** and ketophosphoramide **8b** were synthesized from diethyl-phosphonobutyryl chloride: for **7b**, by an acylation reaction of α-fluoro phosphonoacetic acid in the presence of *n*-BuLi, followed by a decarboxylation reaction[19] and for **8b**, by an amidic coupling with the commercially available diethyl phosphoramidate.

Inhibition Studies and Discussion

Activities of GAPDHs from trypanosome and rabbit muscle were measured using forward and reverse

reactions of the enzymatic process (Scheme 1), as described in the Experimental. The IC_{50} values, shown in Tables 1 and 2, were measured for both organisms, with respect to both substrates in saturating concentrations as described by Misset et al.:[4,5]

1. In the forward reaction, the concentration in GAP was 0.8 mM, largely over the K_m values (150 and 70 μM for Tb and Rm GAPDHs respectively);
2. In the reverse reaction, the concentration in 1,3-diPG may appear lower than the K_m values (100 and 130 μM for Tb and Rm GAPDHs, respectively), owing to the low equilibrium constant[20] in the $3–5\times10^{-4}$ range. However, as shown by Schmidt et al.[21] substrates and products of the PGK catalyzed reaction are strongly associated with the enzyme, the corresponding equilibrium constant being 0.1. In the coupled PGK-GAPDH assay, therefore, the corresponding 1,3-diPG concentration is ten times higher than the part free in solution and thus significantly higher than the K_m value ($\cong 8\,K_m$). This bound 1,3-diPG is then transfered to GAPDH either through a bienzyme complex, or through a fast k_{off} reaction from PGK.[21]

Compound **1e**, having the highest structural homology with 1,3-diPG, proved to be a poor GAPDH inhibitor ($IC_{50} = 2$ mM), totally non-selective either with regard to substrates or with regard to organisms.

Compound **2e**, without a hydroxyl group, was no more selective with respect to substrates, but exhibited a 5-fold higher affinity for the mammalian enzyme than for the parasite enzyme.

Whereas phosphono-phosphates **1e** and **2e** are competitive inhibitors with respect to both substrates, diphosphonates **3d** and **4**, substituted at the C-3 position, are inhibitors only with respect to the 1,3-diPG. Consequently, these differences indicate:

1. that phosphono-phosphates and diphosphonates do not interact at the same binding sites, and
2. that only phosphono-phosphates **1e** and **2e** interact at the GAP binding site.

In addition, comparison of IC_{50} values between compounds **1e**, **3d**, **4** and **5b** clearly shows that the substitution at the C-3 position decreases affinity and supresses selectivity. Chloro-2-oxopentane-1,5-biphosphonic acid **4**[22] had one of the lowest affinities due probably to steric hindrance of the chlorine atom, bulkier than a hydroxyl.

Since a strong homology with the substrate (phosphate group and C-2 substitution) seemed unfavourable to selective activity, all 1,3-diPG analogues designed in the second part (Table 2) were diphosphonates without substitution at the C-3 position.

2-oxo-Diphosphonopentane **5b**, the simplest of these 1,3-diPG analogues, is a selective inhibitor of Tb GAPDH with respect to 1,3-diPG but not the other substrate GAP. No inhibition was observed on Rm GAPDH at a 5 mM inhibitor concentration; the same result was reported in yeast.[23] Surprisingly, **9b**, an analogue of **5b** but bearing a phosphonic acid and a phosphonic ester, has a very close IC_{50} value. It seems that the β-ketophosphonate moiety is the essential ionic frame responsible for the selective inhibitory effect. To improve both affinity and selectivity, a series of chemical modifications of this β-ketophosphonate frame were performed and the inhibitory effects are shown in Table 2.

By introducing one or two fluorine atoms or one nitrogen atom on the β-ketophosphonate moiety, the inhibitory effect on Tb GAPDH was improved without altering selectivity. The IC_{50} values for fluorinated derivatives **6b** and **7b** are in the range of, or lower than, the K_m of 1,3-diPG (100–120 μM); moreover their active concentrations are over-estimated since **6b** and **7b** (racemic compound) exist under their hydrated forms at, respectively 10 and 20%, as evidenced by NMR spectroscopy.

The potential of α-monofluoro or α-difluorophosphonate as stable phosphate mimics has been reported.[18,24] They could therefore act as alternative substrates of the glycolytic enzyme glycerol phosphate dehydrogenase.[25] The introduction of one or two fluorine atoms onto the methylene group increases the acidity of the phosphonate, from 7.6 to 6.5 giving a pK_a value identical to that of the phosphate group. These substitutions also increase the P–C–C angle value from 112 to 116°, restoring the substrate's P–O–C angle[26] (Cambridge data bank). Concerning the β-ketophosphonate, these structural features are less significant since the P–X–C(O) angle is 113° for the mixed anhydride (O)POC(O),[27] 116° for (O)PCC(O)[28,29] and 125° for the amino analogue (O)PNC(O).[30] X-ray 3D structures are not available for α-halogenated-β-ketophosphonate. Since compounds **5b** and **8b**, despite their different P–X–C(O) angles (116 and 125°, respectively),

Table 1. Inhibition studies of Tb and Rm GAPDH with respect to GAP and with respect to 1,3-diPG: IC_{50} values (mM)

	GAPDH Tb		GAPDH Rm	
Compound	/GAP	/1,3-diPG	/GAP	/1,3-diPG
1e	2	2	2	2
2e	0.7	1	0.1	0.2
3d	—[a]	0.5	—[a]	0.5
4	—[a]	1	—[a]	1.5
5b	—[a]	0.35	—[a]	—[a]

[a]No inhibition at 5 mM.

Table 2. Selective inhibition of Tb GAPDH by 1,3-diPG analogues[a]

Compound	IC_{50} (μM)/1,3-diPG[b]
5b	350
9b	300
6b	65
7b	150
8b	200

[a]No effect on the Rm enzyme at 5 mM concentration.
[b]No inhibition with respect to GAP at 5 mM.

have similar affinities for the enzyme, it would seem that the inhibitory effect is not directly linked to geometric parameters.

However, the pK_a values of these molecules[18] show an important difference between their ionisation state in physiological conditions at pH 7.5. From pK_a values of 6.09 for the β-keto-methylphosphonate **5b** and 4.5 for the difluoro-methylphosphonate **6b**, an intermediate pK_a value for the monofluoro analogue **7b** can be assumed. In such cases all compounds are in poly-anionic form inside the active site and the highest affinity is observed for the difluorinated compound **6b**, almost entirely deprotonated. Since the percentage of mono-anionic form at physiological pH is a little higher for compounds **5b** and **7b** than for **6b**, it can be further assumed that the difference in pK_a values could explain, at least partly, the different IC_{50} values for diphosphonates **5b** to **7b**. It is very likely that an additive effect can be ascribed to the restoration of an H-bonding capability between the fluorine atoms and residues in the GAPDH active site. These results clearly demonstrate:

1. for both enzymes, the binding at the GAP site is governed by the presence of the phosphate group rather than the C-3 hydroxy group (compounds **1e** and **2e**); replacement of the latter by an N–OH group leads only to an affinity at the 1,3-diPG binding site;
2. affinities of the 3-(deoxy) compounds at the 1,3-diPG binding site prove to be selective since only observed for the Tb enzyme (compounds **5b** to **8b** Table 2);
3. the affinity for *T. brucei* GAPDH is optimized in compounds where the carboxyphosphonate part has a low pK (under its dianionic form in the active site) and is able to make H-bonds with the protein. Further rationalization and improvements of affinities will require the results of X-ray co-crystallisation, now in progress with Tb GAPDH and compounds **1e**, **2e** and **6b**.

Conclusion

Diversely functionalized 1-phospho,3-oxo,4-phosphonobutanes and 1,5-diphosphono,2-oxopentanes, 1,3-diPG analogues, were synthesized and evaluated as GAPDH inhibitors. Whereas phosphono-phosphates (**1e** and **2e**) and diphosphonates substituted at the C-3 position (**3d** and **4**) proved to inhibit Rm GAPDH as well as Tb GAPDH, all non-substituted diphosphonates (from **5b** to **9b**) are selective inhibitors of Tb GAPDH with respect to 1,3-diPG. The introduction of fluorine or nitrogen atoms on the a β-ketophosphonate improves the affinity of these inhibitors for the parasite enzyme without altering selectivity.

Experimental

Chemistry

General directions. Prior to use, tetrahydrofuran (THF) and diethyl ether (Et_2O) were distilled from sodium benzophenone, dichloromethane (CH_2Cl_2) from P_2O_5 and pyridine or triethylamine from KOH. ^1H, ^{13}C and ^{31}P NMR spectra were obtained on Bruker Instruments AC-250, AC-50 and AC-81 using internal TMS (^1H and ^{13}C) and H_3PO_4 (^{31}P) as references. Chemical shifts (δ), expressed in ppm, are reported for signal centers and coupling constants (*J*) are given in Hz. IR spectra were recorded on a Perkin-Elmer 1600 series FTIR and elemental analyses were performed by the Ecole Nationale Supérieure de Chimie de Toulouse—France. Optical rotations were determined with a Perkin-Elmer 241 polarimeter. Preparative column chromatographies were performed using 70–230 mesh Merck silica gel. HPLC was performed using a hyperprep HS C18 column from Hypersil. The synthesis of compounds **1b**, **1d**, **2b**, **2c**, **2d**, and of all diphosphonates from **5a** to **9a** were performed under an argon atmosphere in dried glassware using freshly distilled solvents.

General synthetic procedure

Methyl ester (2(*R*),3-*O*-isopropylidene) glyceric acid (1a). To a solution of 1,2,5,6-di-*O*-isopropylidene-D-mannitol (10 g, 38.0 mmol) in CH_2Cl_2 (100 mL) sodium periodate (16.2 g, 76 mmol) and water (4 mL) were slowly added. After stirring for 3 h, magnesium sulphate (20 g) was added and the mixture was filtered and evaporated. To the colourless oil a solution of $MeOH/H_2O$, 9:1 (100 mL), sodium hydrogenocarbonate (24 g, 285 mmol) and bromine (7.7 mL, 154 mmol) were slowly added. After stirring overnight at rt, bromine excess was neutralized by sodium thiosulphate and the mixture was extracted with CH_2Cl_2 (2×150 mL). The combined organic layers were dried ($MgSO_4$) and evaporated to give ester **1a** as a colourless oil (9.25 g, 76%).

^1H NMR (250 MHz, $CDCl_3$) δ 1.35 (s, 3H), 1.44 (s, 3H), 3.72 (s, 3H), 4.05 (m, 1H), 4.19 (m, 1H), 4.55 (m, 1H).

^{13}C NMR (50 MHz, $CDCl_3$) δ 25.8, 26.3, 52.4, 67.2, 74.0, 111.3, 171.6.

IR (film): $\nu_{C=O} = 1755\ cm^{-1}$.

Dibenzyl (3(R),4-*O*-isopropylidene, 2-oxobutyl) phosphonate (1b). To an Ar-purged flask containing a solution of dibenzylmethylphosphonate (2.46 g, 8.9 mmol) in THF (15 mL) a 1.6 M solution of butyl-lithium (6 mL, 9.8 mmol) was added at −78 °C. After stirring for 30 min at −78 °C, this mixture was added, via a cannula, to a solution of ester **1a** (1.3 g, 8.1 mmol) in anhydrous THF (15 mL). After stirring for 1 h at −78 °C, the mixture was slowly warmed overnight up to 20 °C. The organic layer was subsequently neutralized with a saturated aqueous solution of NH_4Cl and after THF evaporation, the residue was extracted with AcOEt (3×100 mL). The combined organic layers were dried ($MgSO_4$) and filtered to give phosphonate **1b** (2.95 g, 90%) used without further purification.

^1H NMR (250 MHz, $CDCl_3$) δ 1.21 (s, 3H), 1.28 (s, 3H), 3.97 (m, 2H), 4.40 (m, 1H), 5.08 (m, 4H), 7.36 (m, 10H).

^{13}C NMR (50 MHz, $CDCl_3$) δ 25.0, 26.0, 37.8 (J_{C-P} = 132.2), 65.9, 68.1 (J_{C-P} = 6.1), 80.1 (J_{C-P} = 2.4), 111.2, 127.8, 135.9 (J_{C-P} = 6.2), 202 (J_{C-P} = 6.6).

^{31}P NMR (81 MHz, $CDCl_3$) δ 20.8.

IR (film): $\nu_{C=O}$ = 1724 cm^{-1}; $\nu_{P=O}$ = 1013, 1214 cm^{-1}

Dibenzyl (3(R),4-dihydroxy, 2-oxobutyl) phosphonate (1c). 1 M HCl (5 mL) was added to a solution of phosphonate **1b** (2.7 g, 6.7 mmol) in a mixture of THF/H_2O 1:1 (150 mL). After stirring for 3 days at rt, THF was evaporated off and the aqueous layer extracted with CH_2Cl_2. Evaporation of the solvent and chromatography (CH_2 Cl_2/MeOH, 95:5) yielded **1c** (1 g, 40%).

^{1}H NMR (250 MHz, $CDCl_3$) δ 3.35 (d, 2H, J_{H-P} = 22.4), 3.85 (m, 2H), 4.23 (m, 1H), 5.0 (m, 4H), 7.32 (m, 10H).

^{13}C NMR (50 MHz, $CDCl_3$) δ 38.6 (J_{C-P} = 130.2), 63.5, 68.5 (J_{C-P} = 6.3), 78.6 (J_{C-P} = 1.8), 128.2, 128.8, 135.6 (J_{C-P} = 6.1), 204 (J_{C-P} = 6.4 Hz).

^{31}P NMR (81 MHz, $CDCl_3$) δ 21.6.

IR (film): $\nu_{C=O}$ = 1723 cm^{-1}; $\nu_{P=O}$ = 1019, 1216 cm^{-1}.

Anal calcd for $C_{18}H_{21}O_6P$ (364): C, 59.34; H, 5.81. Found: C, 59.40; H, 5.75.

Dibenzyl (4-dibenzylphosphono, 3-oxo, 2(*R*)-hydroxybutyl) phosphate (1d). Iodine (390 mg, 1.5 mmol) was added at 0 °C to a solution of tribenzylphosphite (560 mg, 1.6 mmol) in anhydrous CH_2Cl_2 (15 mL). After stirring for 10 min at 0 °C and for 15 min at rt, this mixture was added, via a cannula at −30 °C, to a solution of diol **1c** (370 mg, 1.0 mmol) in anhydrous CH_2Cl_2 (20 mL) and pyridine (0.13 mL, 1.5 mmol). After 30 min at rt, the pyridinium salts were filtered off and the organic solution washed with water, dried ($MgSO_4$) and filtered. Evaporation of the solvent and chromatography (CH_2Cl_2/MeOH, 98:2) yielded **1d** (315 mg, 50%) as a colourless oil; $[\alpha]_D$ = +1.8° (*c* 10, MeOH).

^{1}H NMR (250 MHz, $CDCl_3$) δ 3.34 (d, 2H, J = 23.3), 4.26 (m, 3H), 5.02 (dd, 8H, J_{H-H} = 1.6, J_{H-P} = 9.7), 7.32 (m, 20H).

^{13}C NMR (50 MHz, $CDCl_3$) δ 39.1 (J_{C-P} = 128.8), 68.4 (J_{C-P} = 6.2), 69.6 (J_{C-P} = 6.0), 76.5 (J_{C-P} = 6.3), 128.5, 135.6 (J_{C-P} = 6.2), 202.4 (J_{C-P} = 6.3).

^{31}P NMR (81 MHz, $CDCl_3$) δ −0.6, 21.0.

IR (film): $\nu_{C=O}$ = 1725 cm^{-1}; ν_{OH} = 3410 cm^{-1}; $\nu_{P=O}$ = 1015, 1256 cm^{-1}.

Anal. calcd for $C_{32}H_{34}O_9P_2$ (624): C, 61.54; H, 5.49. Found: C, 61.43; H, 5.55.

Dicyclohexylammonium (4-dicyclohexylammoniumphosphono, 3-oxo, 2(*R*)-hydroxybutyl) phosphate (1e). To a H_2-purged flask containing a solution of **1d** (350 mg, 0.56 mmol) in MeOH (70 mL), Pd/C 10% (230 mg) was added. After stirring under a H_2 atmosphere for 30 min at rt, cyclohexylamine (250 μL, 0.56 mmol) was further added and the Pd/C filtered off. Evaporation of MeOH gave tetracyclohexylammonium salt **1e** (370 mg, 100%) as a white solid; $[\alpha]_D$ = −4.6° (*c* 10, MeOH).

Anal. calcd for $C_{28}H_{62}N_4O_9P_2$,4H_2O (732): C, 45.95; H, 9.63; N, 7.65. Found C, 46.01; H, 9.46; N, 7.52.

^{1}H NMR (250 MHz, D_2O) δ 1.7 (m, 40H), 3.1 (m, 4H), 4.1 (m, 2H), 4.5 (m, 1H).

^{13}C NMR (50 MHz, D_2O) δ 26.4, 26.9, 33.0, 47.5 (J_{C-P} = 100.5), 52.8, 67.9 (J_{C-P} = 4.4), 79.7 (J_{C-P} = 7.3), 213.4 (J_{C-P} = 5.4 Hz).

^{31}P NMR (81 MHz, D_2O) δ 4.6, 10.4.

IR (H_2O): $\nu_{C=O}$ = 1701 cm^{-1}; $\nu_{P=O}$ = 1110 cm^{-1}

IR (KBr dispersion): $\nu_{C=O}$ = 1701 cm^{-1}; $\nu_{P=O}$ = 1057 cm^{-1}.

Methyl ester (3-hydroxy) propionic acid (2a). To a solution of NaOH (140 mg, 3.47 mmol) in anhydrous MeOH (17 mL) cooled in an ice bath β-propiolactone (5 g, 69.4 mmol) was slowly added. After stirring for 1 h at rt, the solution was neutralized with concentrated chlorhydric acid and the solvent evaporated. The residue was diluted in CH_2Cl_2 and washed with water. The organic layer was dried ($MgSO_4$), filtered and concentrated to give a colourless oil (5.77 g, 80%).

^{1}H NMR (250 MHz, $CDCl_3$) δ 2.54 (t, 2H), 2.85 (s, 1H, OH), 3.68 (s, 3H), 3.84 (t, 2H).

^{13}C NMR (50 MHz, $CDCl_3$) δ 36.7, 51.8, 58.1, 173.3.

IR (film): $\nu_{C=O}$ = 1730 cm^{-1}.

Methyl ester (3-hydroxy-trimethylsilyl) propionic acid (2b). To a solution of **2a** (2 g, 19.3 mmol) in anhydrous CH_2Cl_2, anhydrous pyridine (1.55 mL, 19.3 mmol) and chlorotrimethylsilane (2.45 mL, 19.3 mmol) were successively added at 0 °C. After stirring for 1 h at 0 °C, the solution was allowed to warm at rt and the solvent evaporated off. Anhydrous Et_2O was then added and the pyridinium salts filtered off. After evaporation, compound **2b** was obtained as a colourless liquid. (3.05 g, 90%).

^{1}H NMR (250 MHz, $CDCl_3$) δ 0.05 (s, 9H), 2.47 (t, 2H), 3.61 (s, 3H), 3.80 (t, 2H).

^{13}C NMR (50 MHz, $CDCl_3$) δ −0.66, 37.6, 51.5, 58.3, 172.1.

Dibenzyl (4-hydroxy, 2-oxobutyl) phosphonate (2c). To a solution of dibenzylmethylphosphonate (3 g, 10.87 mmol), in anhydrous THF (15 mL) at −80 °C, a 1.6 M solution of BuLi (7.5 mL, 12 mmol) was added. After stirring for half an hour at −78 °C, this solution was added, via a cannula, to a solution of ester **2b** (1.9 g, 10.87 mmol) in

anhydrous THF (20 mL) at −78 °C. After 1 h at −78 °C, the mixture was slowly warmed overnight up to 20 °C. The organic layer was then neutralized with a saturated aqueous solution of NH_4Cl and acidified until pH = 1 with a 1 M HCl solution. After THF evaporation, the mixture was extracted with AcOEt (3×100 mL). The combined organic layers were then dried on $MgSO_4$, filtered, and evaporated to give 3.9 g of a pale yellow oil. Purification by chromatography (CH_2Cl_2/MeOH, 98:2) yielded **2c** as a colourless oil (2.84 g, 75%).

^{1}H NMR (250 MHz, $CDCl_3$) δ 2.73 (t, 2H), 3.37 (d, 2H, J_{H-P} = 22.3), 3.51 (s, 1H, OH), 3.77 (t, 2H), 4.94–5.06 (m, 4H), 7.29 (m, 10H).

^{13}C NMR (50 MHz, $CDCl_3$) δ 42.9 (d, J_{P-C} = 128.3), 46.6, 57.5, 68.1 (d, J_{P-C} = 6.3), 128.4, 135.6, 202.1 (d, J_{P-C} = 6).

^{31}P NMR (81 MHz, $CDCl_3$) δ 21.2.

Dibenzyl (4-dibenzylphosphono, 3-oxobutyl) phosphate (2d). A quantity of iodine (1.08 g, 4.2 mmol) was added at 0 °C to a solution of tribenzylphosphite (1.66 g, 4.71 mmol) in anhydrous CH_2Cl_2 (15 mL). After stirring for 10 min at 0 °C and for 5 min at rt, this solution was added, via a cannula, to a solution of alcohol **2c** (820 mg, 2.35 mmol) and anhydrous pyridine (0.36 mL, 4.2 mmol) in anhydrous CH_2Cl_2 (15 mL) at 0 °C.

After stirring for 30 min at 0 °C and for a further 30 min at rt, the pyridinium salts were filtered off, the organic layer was washed with water, dried ($MgSO_4$) and filtered. Purification by HPLC on C18 silica gel (CH_3CN–H_2O 60:40) led to **2d** as a colourless oil (2.14 g, 75%).

^{1}H NMR (250 MHz, $CDCl_3$) δ 2.83 (t, 2H), 3.04 (d, 2H, J_{H-P} = 22.7), 4.18 (td, 2H), 5.01 (m, 8H), 7.32 (m, 20H).

^{13}C NMR (50 MHz, $CDCl_3$) δ 42.9 (d, J_{C-P} = 117.6), 44.0 (d, J_{C-P} = 2.5), 62.0 (d, J_{C-P} = 5.4), 68.2 (d, J_{C-P} = 6.4), 69.4 (d, J_{C-P} = 5.5), 127.5–128.7, 135.7 (m), 198.3 (d, J_{C-P} = 6.35).

^{31}P NMR (81 MHz, $CDCl_3$) δ −1.13, 20.41.

Dicyclohexylammonium (4-dicyclohexylammoniumphosphono, 3-oxo) phosphate (2e). To an H_2-purged flask containing a solution of **1d** (114 mg, 0.19 mmol) in MeOH (20 mL), Pd/C 10% (100 mg) was added. After stirring under a H_2 atmosphere for 30 min at rt, cyclohexylamine (81 μL, 0.75 mmol) was added and Pd/C filtered off. Evaporation of MeOH gave tetracyclohexylammonium salt **2e** as a white amorphous powder. (116 mg, 97%).

^{13}C NMR (50 MHz, D_2O) δ 26.6 (m), 31.4 (m), 33.0 (m), 41.7, 52.3(d, J_{C-P} = 74.7), 52.8, 60.2 (d, J_{C-P} = 12.4), 211.0 (d, J_{C-P} = 5.3).

^{31}P NMR (81 MHz, D_2O) δ 2.28, 10.17.

Anal. calcd for $C_{28}H_{62}N_4O_8P_2$,5H_2O (734): C, 45.76; H, 9.87; N, 7.62. Found C, 45.98; H, 8.36; N, 7.72.

Diethyl (ethyl, 2-*O*-benzylhydroxylamino) phosphonate (3a). To a solution of *O*-benzyl-hydroxylamine (2.6 g, 21.1 mmol) in anhydrous methanol (25 mL), diethylvinylphosphonate (1.1 mL, 7.1 mmol) was added. After stirring for 4 days at 60 °C, CH_2Cl_2 (200 mL) was added, and the mixture washed with water (40 mL). The organic layer was dried ($MgSO_4$), filtered and evaporated. After purification by chromatography (AcOEt), **3a** was obtained as a colourless oil (1.12 g, 55%)

^{1}H NMR (250 MHz, $CDCl_3$) δ 1.26 (td, 6H), 1.98 (td, 2H, J_{H-P} = 18.3), 3.14 (m, 2H), 4.04 (qd, 4H), 4.65 (s, 2H), 7.29 (m, 5H).

^{13}C NMR (50 MHz, $CDCl_3$) δ 16.4 (d, J_{C-P} = 6), 24.0 (d, J_{C-P} = 139.6), 45.8 (d, J_{C-P} = 2.8), 61.6 (d, J_{C-P} = 6.3), 76.1, 128.1, 137.8.

^{31}P NMR (81 MHz, $CDCl_3$) δ 30.3.

Dimethyl-phosphono-acetic acid (3b). To a solution of NaOH (444 mg, 11.1 mmol) in water (20 mL), trimethylphosphono-acetate (2.01 g, 11.1 mmol) was added. After stirring for 1 h, the aqueous layer was washed with AcOEt (20 mL) and evaporated to give **3b** as a white powder. (2.07 g, 98%)

^{1}H NMR (250 MHz, D_2O) δ 2.78 (dd, 2H, J_{H-P} = 21.5), 3.75 (d, 3H, J_{H-P} = 11.2), 3.76 (d, 3H, J_{H-P} = 11.2).

^{13}C NMR (50 MHz, D_2O) δ 40.2 (d, J_{C-P} = 121.4), 56.1 (d, J_{C-P} = 6.5).

^{31}P NMR (81 MHz, $CDCl_3$) δ 31.1.

(*N*-ethyldiethylphosphonate, *N*-1-oxoethyldimethylphosphonate) *O*-benzylhydroxylamine (3c). To a solution of **3b** (532 mg, 2.8 mmol) in a mixture THF–H_2O 1/1 (60 mL), **3a** (803 mg, 2.8 mmol) was added. The solution was acidified to pH = 4 with 1 M HCl and *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (537 mg, 2.8 mmol) was added. After stirring overnight at rt, THF was evaporated. Compound **3c** was extracted with AcOEt (3×100 mL), washed with saturated aqueous $NaHCO_3$, dried on $MgSO_4$, filtered and evaporated. Compound **3c** was obtained in a pure state, no further purification being necessary (1.05 g, 86%).

^{1}H NMR (250 MHz, $CDCl_3$) δ 1.05 (td, 6H), 1.86 (m, 2H), 2.84 (d, 2H, J_{H-P} = 21.8), 3.51 (d, 6H, J_{H-P} = 7.4), 3.59 (m, 2H), 3.82 (qd, 4H), 4.68 (s, 2H), 7.13 (m, 5H).

^{13}C NMR (50 MHz, $CDCl_3$) δ 16.1, 22.7 (d, J_{C-P} = 139.1), 30.9 (d, J_{C-P} = 136.6), 40.0, 52.9, 61.6, 76.5, 128.9, 133.8, 166.6.

^{31}P NMR (81 MHz, $CDCl_3$) δ 23.6, 27.6.

(*N*-ethyldiethylphosphonate, *N*-1-oxoethyldimethylphosphonate) hydroxylamine (3d). To an H_2-purged flask containing a solution of **3c** (270 mg, 0.62 mmol) in MeOH (70 mL), 10% Pd/C (40 mg) was added. After

stirring for 2 h at rt, Pd/C was filtered off and MeOH evaporated to give **3d** as a colourless oil (210 mg, 98%).

^{1}H NMR (250 MHz, $CDCl_3$) δ 1.20 (t, 6H), 2.00 (m, 2H), 3.10 (d, 2H, J_{H-P} = 21.4), 3.67 (d, 6H, J_{H-P} = 11.0), 3.79 (m, 2H), 3.98 (m, 4H).

^{13}C NMR (50 MHz, $CDCl_3$) δ 16.2 (d, J_{C-P} = 5.0), 21.5 (d, J_{C-P} = 93.7), 31.2 (d, J_{C-P} = 134.9), 42.4, 53.2 (d, J_{C-P} = 5.7), 62.1 (d, J_{C-P} = 5.7), 164.9.(d, J_{C-P} = 6.5)

^{31}P NMR (81 MHz, $CDCl_3$) δ 25.5, 29.6.

(*N*-ethyldihydroxyphosphonate, *N*-1-oxoethyldihydroxyphosphonate) hydroxylamine, tetra-sodium salt (3e). To an Ar-purged flask containing **3d** (210 mg, 0.61 mmol), bromotrimethylsilane (0.66 mL, 5 mmol) was added. After stirring overnight at rt, bromotrimethylsilane and ethyl-bromide excesses were evaporated, the residue being diluted in Et_2O (15 mL) and the tetra-hydroxydiphosphonate extracted with water (2×25 mL). After neutralization until pH = 7.5 with 1 M NaOH and lyophilization, **3e** was obtained as a white powder (207 mg, 97%).

^{1}H NMR (250 MHz, D_2O) δ 1.79–1.92 (m, 2H), 2.78 (d, 2H, J_{H-P} = 19.4), 3.69–3.78 (m, 2H).

^{13}C NMR (50 MHz, D_2O) δ 28.7 (d, J_{C-P} = 128.8), 39.2 (d, J_{C-P} = 110.2), 46.4, 172.5 (d, J_{C-P} = 7.3).

^{31}P NMR (81 MHz, D_2O) δ 15.3, 21.1.

Tetraethyl 2-oxopentane-1,5-bisphosphonate (5a). To a solution of diethylmethylphosphonate (304 mg, 2 mmol), in anhydrous THF (15 mL) at −78 °C, a 1.6 M solution of BuLi (1.25 mL) was added. After stirring for half an hour at −78 °C, this solution was added, via a cannula, to a solution of triethyl 4-phosphonobutyrate (505 mg, 2 mmol) in anhydrous THF (20 mL) at −78 °C. After 1 h at −78 °C, the mixture was slowly warmed overnight up to 20 °C. The organic layer was then neutralized with a saturated aqueous solution of NH_4Cl and the mixture extracted with AcOEt (3×100 mL). The combined organic layers were subsequently dried on $MgSO_4$, filtered, and the solvent evaporated off to give a yellow oil. The bisphosphonate 5a was isolated by flash chromatography (CH_2Cl_2/MeOH, 97:3) as a colourless oil (548 mg, 77%).

^{1}H NMR (250 MHz, $CDCl_3$) δ 1.98 (t, 12H), 1.55–1.90 (m, 4H), 2.85 (t, 2H), 2.95 (d, 2H, J_{H-P} = 22.8), 3.95 (q, 8H).

^{13}C NMR (50 MHz, $CDCl_3$) δ 16.3, 16.5, 24.4 (d, J_{C-P} = 141.3), 42.5 (d, J_{C-P} = 142.1), 43.7, 61.5 (d, J_{C-P} = 6.5), 62.5 (d, J_{C-P} = 6.3), 201.0 (d, J_{C-P} = 6.0).

^{31}P NMR (81 MHz, $CDCl_3$) δ 19.8, 31.3.

Mass spectrometry (DCI, NH_3) *m*/*z* 395 [MH^+], 412 [MNH_4^+].

2-Oxopentane-1,5-bisphonic acid (5b).[18] Bromotrimethylsilane (1.41 mL, 11.2 mmol) was added dropwise to ester **5a** (534 mg, 1.5 mmol) under argon. After stirring for 1 night at rt, the volatiles were evaporated in vacuo and the residue dissolved in Et_2O. The bisphosphonic acid was then extracted in water (2×10 mL) and the aqueous layer titrated to pH 7.1 with 1 M NaOH. Lyophilization provided the title compound as a hygroscopic white solid (477 mg, 95%).

^{1}H NMR (250 MHz, D_2O) δ 1.25–1.65 (m, 4H), 2.60 (t, 2H), 2.75 (2H, d, J_{H-P} = 22.0).

^{13}C NMR (50 MHz, D_2O) δ 18.2, 27.7 (d, J_{C-P} = 131.9), 44.1 (d, J_{C-P} = 12.5), 47.1 (d, J_{C-P} = 104.1), 210.0 (d, J_{C-P} = 15.9).

^{31}P NMR (81 MHz, D_2O) δ 11.5, 25.7.

Tetraethyl 1,1-difluoro-2-oxopentane-1,5-bisphosphonate (6a). To a solution of LDA (3.9 mL, 2 M solution in hexane) in anhydrous THF (2 mL) at −78 °C, a solution of diethyl-difluoromethylphosphonate (1.45 g, 7.73 mmol) in THF (2 mL) was added. After stirring for 30 min at −78 °C, a solution of triethyl 4-phosphonobutyrate (1.5 g, 5.95 mmol) in THF (2 mL) was slowly added, via a cannula, and the reaction mixture was maintained for 1 h at −78 °C. Subsequently, glacial AcOH (0.78 mL) and a saturated solution of NH_4Cl (15 mL) were successively added. The bisphophonate was extracted with AcOEt (3×100 mL), the combined organic layers were dried ($MgSO_4$), filtered and the solvent evaporated. Purification by flash chromatography (CH_2Cl_2/MeOH, 97:3) led to compound **6a** as a colourless oil (1.69 g, 72%).

^{1}H NMR (250 MHz, $CDCl_3$) δ 1.25 (t, 12H), 1.56–1.90 (m, 4H), 2.81 (t, 2H), 3.97 (m, 4H), 4.16 (m, 4H).

^{13}C NMR (50 MHz, $CDCl_3$) δ 15.8, 16.3, 24.5 (d, J_{C-P} = 141.8), 37.7 (d, J_{C-P} = 15.3), 61.5 (d, J_{C-P} = 6.4), 65.5 (d, J_{C-P} = 6.6), 112.9 (td, J_{C-P} = 195.2, J_{C-F} = 273.4), 198.4 (dt, J_{C-P} = 14.1, J_{C-F} = 23.4).

^{31}P NMR (81 MHz, $CDCl_3$) δ 3.05 (t, J_{P-F} = 97), 30.7.

1,1-Difluoro-2-oxopentane-1,5-bisphosphonic acid (6b).[18] Bromotrimethylsilane (2 mL, 16 mmol) was added dropwise to ester **6a** (886 mg, 2.25 mmol) under argon. After stirring for 3 days at rt, the volatiles were evaporated in vacuo and the residue dissolved in Et_2O. The bisphosphonic acid was then extracted in water (2×10 mL) and the aqueous layer titrated to pH 7.1 with 1 M NaOH. Lyophilization gave the title compound as a hygroscopic white solid (816 mg, 98%).

^{1}H NMR (250 MHz, D_2O) δ 1.45–1.85 (m, 4H), 2.90 (m, 2H)

^{13}C NMR (50 MHz, D_2O) δ 19.3, 29.6 (d, J_{C-P} = 132.1), 38.5 (m), 120.7 (td, J_{C-P} = 158.7, J_{C-F} = 269.5), 206.8 (m).

^{31}P NMR (81 MHz, D_2O) δ 2.7 (t, J_{P-F} = 98), 25.7.

Tetraethyl 1,fluoro-2-oxopentane-1,5-bisphosphonate (7a). To a solution of diethyl,4-phosphonobutyric acid (2 g,

8.9 mmol) in anhydrous CH_2Cl_2 (20 mL), $SOCl_2$ (0.72 mL, 9.7 mmol) was added. After stirring for 4 h at rt, the solvent was evaporated off and the oily residue dissolved in anhydrous THF (20 mL).

To a solution of diethyl,1-fluoro-phosphonoacetic acid (1.63 g, 7.6 mmol) in anhydrous THF (25 mL), a 1.6 M solution of BuLi (10.4 mL, 16.7 mmol) was added at −78 °C. After stirring for 30 min at −78 °C, the solution of acid chloride was added, via a cannula, to the carbanion. After 1 h at −78 °C, the solution was allowed to warm overnight up to rt, neutralized with a saturated solution of NH_4Cl and extracted with CH_2Cl_2 (2×100 mL). The organic layers were dried ($MgSO_4$), filtered and evaporated off to give a yellow oil. Purification by chromatography (CH_2Cl_2/MeOH, 97:3) led to **7a** as a colourless oil (570 mg, 20%).

^{1}H NMR (250 MHz, $CDCl_3$) δ 1.28 (t, 12H), 1.63–1.92 (m, 4H), 2.74 (t, 2H), 3.96–4.22 (m, 8H), 5.09 (dd, 1H, J_{H-P} = 14.1, J_{H-F} = 47.8).

^{13}C NMR (50 MHz, $CDCl_3$) δ 15.8 (d, J_{C-P} = 2.7), 16.3, 24.5 (d, J_{C-P} = 141.6), 38.9 (d, J_{C-P} = 15.1), 61.5 (d, J_{C-P} = 6.5), 64.2 (d, J_{C-P} = 5.2), 91.5 (dd, J_{C-P} = 152.7, J_{C-F} = 197.6), 202.0 (d, J_{C-P} = 19.6).

^{31}P NMR (81 MHz, $CDCl_3$) δ 10.2 (d, J_{P-F} = 71), 31.1.

1,Fluoro-2-oxopentane-1,5-bisphosphonic acid (7b). Bromotrimethylsilane (1 mL, 8 mmol) was added dropwise to ester **7a** (300 mg, 0.80 mmol) under argon. After stirring for 3 days at rt, the volatiles were evaporated in vacuo and the residue dissolved in Et_2O. The bisphosphonic acid was then extracted in water (2×15 mL) and the aqueous layer titrated to pH 7.1 with 1 M NaOH. Lyophilization supplied the title compound as a hygroscopic white solid (273 mg, 97%).

^{1}H NMR (250 MHz, D_2O) δ 1.45–1.60 (m, 2H), 1.64–1.80 (m, 2H), 2.62–2.85 (m, 2H), 5.18 (dd, J_{H-P} = 13.9, J_{H-F} = 48.2).

^{13}C NMR (50 MHz, D_2O) δ 19.8 (d, J_{C-P} = 16.0), 29.8 (d, J_{C-P} = 132.6), 42.1 (d, J_{C-P} = 17.2), 65.3 (d, J_{C-P} = 6.2), 99.5 (dd, J_{C-P} = 125.8, J_{C-F} = 188.0), 213.0 (d, J_{C-P} = 14.7).

^{31}P NMR (81 MHz, D_2O) δ 5.9 (d, J_{P-F} = 58.8), 25.9; 7.4 (d, J_{P-F} = 63.6), 25.7.

^{19}F NMR (188 MHz, D_2O) δ −123.2 (dd, J_{F-P} = 60.1, J_{F-H} = 48.5), −126.5 (dd, J_{F-P} = 64.0, J_{F-H} = 48.3).

Diethyl, 1-oxo,4-diethylphosphonobutane phosphoramidate (8a). To a solution of diethyl, 4-phosphonobutyric acid (530 mg, 2.36 mmol) in anhydrous Et_2O (20 mL), oxalyl chloride (0.4 mL, 3.06 mmol) was added. After refluxing for 1 night, the solution was neutralized with anhydrous triethylamine and a solution of diethylphosphoramidate (350 mg, 2.30 mmol) in anhydrous Et_2O (20 mL) was added. Anhydrous Et_3N (0.32 mL, 2.3 mmol) was then added, the solution refluxing for 4 days. The triethylammonium salts were subsequently filtered off, the organic layer washed with water (10 mL), dried ($MgSO_4$), filtered off and evaporated in vacuo. Purification by flash chromatography (CH_2Cl_2/MeOH, 95:5) led to **8a** (206 mg, 25%).

^{1}H NMR (250 MHz, $CDCl_3$) δ 1.21–1.35 (m, 12H), 1.71–1.94 (m, 4H), 2.45 (t, 2H), 3.98–4.22 (m, 8H).

^{13}C NMR (50 MHz, $CDCl_3$) δ 16.1 (d, J_{C-P} = 6.8), 16.5 (d, J_{C-P} = 6.0), 17.9 (d, J_{C-P} = 4.5), 24.8 (d, J_{C-P} = 141.2), 34.1 (d, J_{C-P} = 16.1), 61.6 (d, J_{C-P} = 6.5), 64.1 (d, J_{C-P} = 5.8), 174 (d, J_{C-P} = 3.3).

^{31}P NMR (81 MHz, $CDCl_3$) δ −2.2, 31.3.

1-oxo,4-phosphonobutane phosphoramidate (8b). Bromotrimethylsilane (0.65 mL, 5.2 mmol) was added dropwise to ester **8a** (310 mg, 0.86 mmol) under argon. After stirring for 3 h at rt (^{31}P NMR: −20.9, 21.9), the volatiles were evaporated in vacuo and the residue dissolved in acetone. Water (0.1 mL) was added and the solution stirred for half an hour. Evaporation in vacuo of solvents led to pure **8b** (198 mg, 93%).

^{1}H NMR (250 MHz, D_2O) δ 1.48–1.79 (m, 4H), 2.31 (t, 2H).

^{13}C NMR (50 MHz, D_2O) δ 20.1 (d, J_{C-P} = 3.9), 27.9 (d, J_{C-P} = 135.3), 36.4 (d, J_{C-P} = 17.5), 180.1.

^{31}P NMR (81 MHz, D_2O) δ 0.6, 31.6.

IR (film): $\nu_{C=O}$ = 1816.

Mass spectrometry (DCI, CH_4) m/z 248 [MH^+].

Dibenzyl(2-oxo,5-diethylphosphono) phosphonate 9a. To a solution of dibenzylmethylphosphonate (1.1 g, 4 mmol), in anhydrous THF (25 mL) at −78 °C, a 1.6 M solution of *n*-BuLi (2.5 mL) was added. After stirring for 30 min at −78 °C, this solution was added, via a cannula, to a solution of triethyl 4-phosphonobutyrate (1.0 g, 4 mmol) in anhydrous THF (30 mL) at −78 °C. After 1 h at −78 °C, the mixture was slowly warmed overnight up to 20 °C. The organic layer was then neutralized with a saturated aqueous solution of NH_4Cl and the mixture extracted with AcOEt (3×150 mL). The combined organic layers were then dried on $MgSO_4$, filtered, and the solvent evaporated off to give a yellow oil. The bisphosphonate **9a** was isolated by flash chromatography (CH_2Cl_2/MeOH, 97:3) as a colourless oil (1.6 g, 85%).

^{1}H NMR (250 MHz, $CDCl_3$) δ 1.23 (t, 6H), 1.52–1.78 (m, 4H), 2.60 (t, 2H), 3.00 (d, 2H, J_{H-P} = 22.7), 3.94–4.06 (m, 4H), 4.88–5.05 (m, 4H), 7.28 (m, 10H).

^{13}C NMR (50 MHz, $CDCl_3$) δ 16.4 (d, J_{C-P} = 6.9), 16.5, 24.4 (d, J_{C-P} = 141.1), 42.6 (d, J_{C-P} = 128.2), 43.9 (d, J_{C-P} = 14.8), 61.4 (d, J_{C-P} = 6.5), 68.0 (d, J_{C-P} = 6.3), 128.1, 128.6, 135.7 (d, J_{C-P} = 6.2), 200.6 (d, J_{C-P} = 5.6).

^{31}P NMR (81 MHz, $CDCl_3$) δ 20.9, 31.3.

Dicyclohexylammonium (2-oxo,5-diethylphosphono) phosphonate 9b. To an H_2-purged flask containing a solution of **9a** (1.0 g, 2.1 mmol) in MeOH (30 mL), Pd/C 10% (100 mg) was added. After stirring under an H_2 atmosphere for 30 min at rt, cyclohexylamine (450 μL, 4.2 mmol) was added and Pd/C filtered off. Evaporation of MeOH gave tetracyclohexylammonium salt **2e** as a white amorphous powder (1.0 g, 98%).

^{13}C NMR (50 MHz, CD_3OD) δ 16.8 (d, $J_{C-P}=6.0$), 17.6 (d, $J_{C-P}=4.5$), 25.2 (d, $J_{C-P}=140.1$), 25.6, 26.1, 32.6, 51.2, 63.1 (d, $J_{C-P}=6.4$), 209.3.

^{31}P NMR (81 MHz, CD_3OD) δ 11.3, 34.7.

Inhibition studies

Rabbit muscle glyceraldehyde-3-phosphate-dehydrogenase (EC 1.2.1.12), yeast phosphoglycerate kinase (EC 2.7.2.3), and all substrates and cofactors were purchased from Sigma.

Inactivation studies

In the different coupled assays shown in the following inactivation study, the enzyme activities were measured using either the forward or the reverse direction. The reactions were monitored by absorbance decrease or increase (at 340 nm) of NADH; its concentration was calculated from this absorbance using the value $\varepsilon_{340}=6.22\,mM^{-1}\,cm^{-1}$. All inhibitors were tested by preincubation for 5 min with the enzyme in 0.1 M triethanolamine buffer (pH 7.5 and ionic strength 0.15) at 25 °C. The enzymatic reaction was initiated by the addition of the reaction mixture in accordance with the direction of the reaction followed.

GAPDH inactivation

1. With respect to glyceraldehyde-3-phosphate (GAP) the reaction mixture (1 mL) contained buffer, 1 mM EDTA, 2 mM NAD+, 0.8 mM GAP, 0.1 M KCl and 10 mM potassium phosphate.
2. With respect to 1,3-diPG, a linked assay system was used in which the reaction of GAPDH is the reverse of that of the glycolytic pathway. The assay mixture (1 mL) contained buffer, 1 mM EDTA, 5.6 mM 3-PG, 1 mM ATP, 5 mM $MgSO_4$, 0.42 mM NADH and a saturating level of yeast PGK (11units).

The inhibitor concentration required for 50% inhibition (IC_{50}) was calculated from at least five inhibitor concentrations which were tested at substrate and cofactor saturating conditions. The percentage of remaining activity was calculated by comparison with an inhibitor-free control experiment.

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