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New inhibitors of rabbit muscle triose-phosphate isomerase

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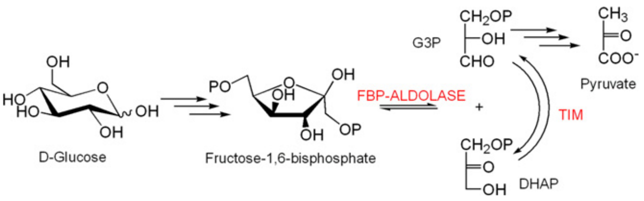
Abstract—We describe the synthesis and evaluation of three new competitive inhibitors of triose-phosphate isomerase. One of them (phosphoglycoloamidoxime: $K_i = 4.5$ µM) is among the best reversible inhibitors so far reported for this enzyme.

1. Introduction

Triose-phosphate isomerase (TIM; EC 5.3.1.1.) catalyzes the reversible interconversion of dihydroxyacetone phosphate (DHAP) and glyceraldehyde phosphate (G3P). Only G3P can proceed further down the glycolytic pathway (Scheme 1). It is one of the most studied enzymes.



Scheme 1. Fructose-bisphosphate aldolase (FBP-aldolase) and TIM: two central enzymes of the glycolysis.

TIM catalytic mechanism is for a large part understood, and has even been considered as a model for enzyme efficiency knowledge.[1] Tertiary structures of TIM from different sources have been resolved[2] (*Plasmodium*, rabbit, yeast, chicken, *Trypanosoma*, *Leishmania*, *Bacillus*, human, etc.). Surprisingly, only a few inhibitors of TIM have been reported: halo-acetone phosphate[3] and glycidol phosphate[4] were used as irreversible inactivators to label active site residues. 3-Phosphoglycerate, glycerol phosphate and phosphoenol pyruvate are relatively weak reversible inhibitors ($K_i = 0.2$–1.3 mM). Phosphoglycolohydroxamate[5] **1a** and phosphoglycolic acid[6] are the best competitive inhibitors, with K_i values of 6–14 and 3 µM, respectively, against the rabbit muscle enzyme. These two compounds, in spite of their relatively low affinity for the enzyme (K_m/K_i around 300), have been considered as 'high-energy intermediate (HEI) analogue' and 'transition-state analogue', respectively[7] (Fig. 1). All these compounds are active on TIM from different sources. Only one hexapeptidic inhibitor shows a marked selectivity ($K_i = 3$ µM) against the *Trypanosoma* enzyme.[8]

The synthesis and testing of new inhibitors of TIM are of interest for the main following reasons:

- Most of the crystal structures so far reported have been obtained on TIM-inhibitor complexes. For future new structures to be resolved, other inhibitors will be welcome.
- Triose-phosphate isomerase is a ubiquitous glycolytic enzyme. However, it could be considered as a potential therapeutic target if significant differences in

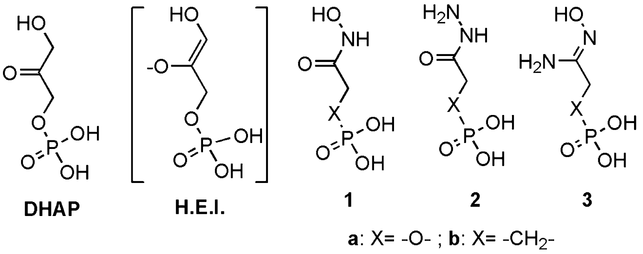


Figure 1. Substrate, high-energy intermediate (HEI) and synthesized products.

Keywords: Triose-phosphate isomerase; Class II fructose-bisphosphate aldolase; Enzyme inhibitor; Phosphoglycoloamidoxyme; Phosphoglycolohydrazide; Phosphonate inhibitors.

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structures are found between human TIM and the enzyme from different pathogenic species. Selective inhibition of the enzyme present in microorganisms such as *Plasmodium*,[9,10] *Leishmania*[11] or *Trypanosoma*,[8,12] whose growth depends strongly or exclusively on glycolysis is of special interest and has already been considered.

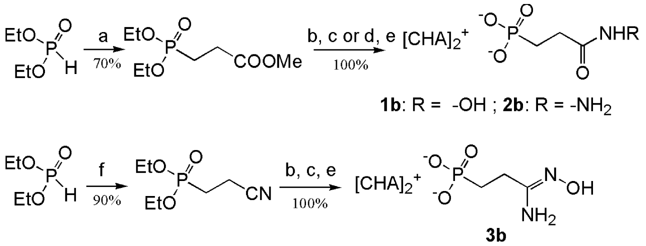
- We recently published the synthesis and evaluation of two new selective inhibitors of class II FBP-aldolase: phosphoglycolohydrazide **2a** and phosphoglycoloamidoxime **3a** ($K_i = 0.34$ and 2.3 μM against yeast aldolase, respectively).[13] These compounds are very weak inhibitors of the mammalian FBP-aldolase. Since class II aldolase is present only in microorganisms (among which many pathogenic bacteria and yeasts), we consider these inhibitors as potential synthetic antibiotics. This is realistic only if other mammalian glycolytic enzymes (like TIM) are not significantly inhibited by these products.

We report hereby the synthesis of **2a**, **3a**, and of their corresponding phosphonates analogues **2b**, **3b**, and their testing as inhibitors of a mammalian TIM. We also prepared and evaluated compound **1b** (the phosphonate analogue of the old known phosphoglycolohydroxamate **1a**,[5]), which was briefly described only recently.[14]

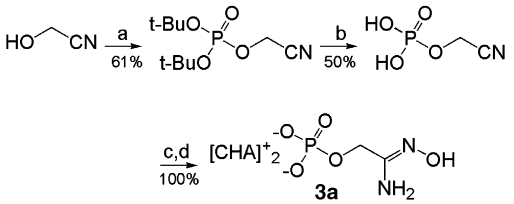
As shown in Figure 1, these products are (at least) analogues of the substrate DHAP, or (at best) analogues of the intermediate ene-diol(ate).

2. Synthesis

The phosphonates **1**–**3b** were prepared[15] according to Scheme 2.



Scheme 2. Original synthesis of phosphonate inhibitors. Reagents: (a) methyl acrylate, DBU; (b) TMS–Br; (c) NH_2OH; (d) $NH_2–NH_2$; (e) cyclohexylamine (CHA) (or: Dowex 50 CHA^+); (f) acrylonitrile, DBU.



Scheme 3. New synthesis of phosphoglycoloamidoxime **3a**. Reagents: (a) $(^tBuO)_2P(O)Cl$, NaH, DMF; (b) Amberlite 15, CH_3CN; (c) NH_2OH; (d) cyclohexylamine (CHA).

Compound **2a** was synthesized according to our previously described method.[16] Phosphoglycoloamidoxime **3a** was prepared according to Scheme 3,[17] by a simplified procedure as compared to our previously described preparation.[13]

3. Results and discussion

Enzymatic assays: the formation of DHAP from G3P was followed spectrophotometrically (340 nm) by measuring the consumption of NADH in a coupled system employing TIM and a large excess of glycerol-3-phosphate dehydrogenase, according to established procedures[19] (the concentration of G3P in the assay was estimated enzymatically with the same coupled system). Not surprisingly, the two phosphonates **2b** and **3b** were very poor inhibitors of TIM (Table 1). Only **1b** showed a significant inhibition, with a K_i value of 145 μM (to be compared with a published value of $K_i = 3$ μM for the phosphate **1a**).[5a] This confirms that a phosphoester oxygen is required for an efficient binding of inhibitors (substrates) into the active site of TIM. Occasionally, the same conclusion was deduced from our parallel experiments with FBP-aldolases (Table 1).

On another hand, the two phosphates **2a** and **3a** were more effective inhibitors of TIM, with K_i values of 111 and 4.5 μM, respectively. Thus, **3a** is as effective against TIM as the old known phosphoglycolohydroxamate **1a**. However, one major advantage of **3a** over **1a** is its increased stability towards hydrolysis: the amidoxime remained unchanged after 72 h in water at room temperature, while the hydroxamate was for part hydrolyzed into the corresponding acid (30% as estimated by ^{13}C NMR). This last point is of importance in view of a potential utilization of amidoximes as drugs, since the release of hydroxylamine in vivo by hydrolysis would be problematic.

The three tested inhibitors **2a**, **3a**, **1b** give (almost) purely competitive inhibition patterns (Figs. 2 and 3).

Of special interest the comparison between the activities of the two inhibitors **2a** and **3a** on TIM and FBP-aldolase (Table 1): phosphoglycolohydrazide **2a** is a good inhibitor of class II aldolases, with an only modest activity on TIM, while phosphoglycoloamidoxime **3a** has comparable performances on the two enzymes. This means that **2a** (or a prodrug derivative) could inhibit the class II aldolase (e.g., from a pathogenic bacterium)

Table 1. Results of inhibition tests

	Class II FBP-aldolase		TIM	
	IC_{50}	K_i	IC_{50}	K_i
1a[a]	—	*0.01*	—	*3*
2a	—	0.34 ± 0.15	—	111 ± 38
3a	—	2.3 ± 0.19	—	4.5 ± 0.6
1b	—	145 ± 15	—	159 ± 13
2b	3632 ± 54	—	27,000 ± 4553	—
3b	3420 ± 73	—	9600 ± 2937	—

[a] Data from Ref. 5a.

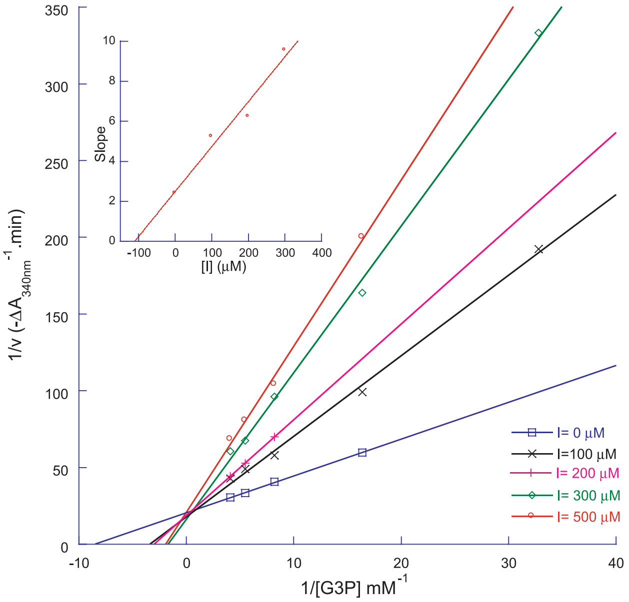


Figure 2. Inhibition of TIM by phosphoglycolohydrazide **2a**.

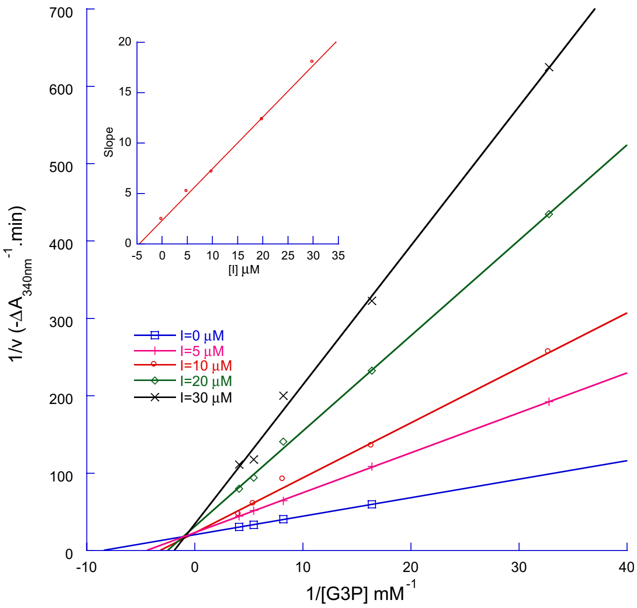


Figure 3. Inhibition of TIM by phosphoglycoloamidoxime **3a**.

in vivo without affecting neither the class I aldolase nor the TIM of the (mammal) host.

From a mechanistic point of view, it is well established that TIM proceeds via the formation of an ene-diolate (rather than an ene-diol).[18] The phosphoglycolohydroxamate **1a** has been considered by Collins[5a] to act through its tautomeric form as a stable analogue of an ene-diol (Fig. 4).

Considering the pK_a value of the hydroxamic acid function (around 9.6), other authors have estimated that **1a** can be deprotonated in the active site of TIM, and thus could mimic the ene-diolate high-energy intermediate.[7] Similarly, the amidoxime **3a** can be thought to be active

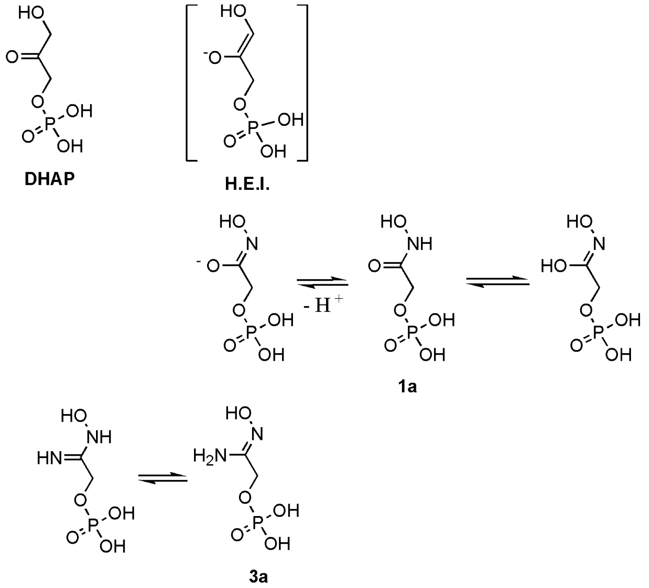


Figure 4. Tautomeric forms of **1a**, deprotonated **1a** and **3a**, compared to the substrate DHAP and to the enolate intermediate HEI.

either as a substrate analogue of DHAP or as a stable analogue of the ene-diol(ate) reaction intermediate. Since the amidoxime **3a** cannot be deprotonated at pH 7.9, and since the inhibitory properties of **1a** and **3a** are quite similar (and much too low for these compounds to be considered as transition-state analogues), one can conclude that the two products are bound into the active site of TIM in the same protonated tautomeric form.

In conclusion, we have devised the synthesis and evaluation of new competitive inhibitors of rabbit muscle triose-phosphate isomerase. These compounds should also be tested on TIM from different origins, especially from pathogenic organisms.

References and notes

1. Knowles, J. R. *Nature* **1991**, *350*, 121–124.
2. (a) Parthasarathy, S.; Eaazhisai, K.; Balaram, H.; Balaram, P.; Murthy, M. R. N. *J. Biol. Chem.* **2003**, *278*, 52461–52470; (b) Aparicio, R.; Fereira, S. T.; Leite, N. R.; Polikarpov, I. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **2000**, *D56*, 1492–1494; (c) Wieranga, R. K.; Noble, M. E.; Davenport, R. C. *J. Mol. Biol.* **1992**, *224*, 1115–1126; (d) Lambeir, A.-M.; Backman, J.; Ruiz-sanz, J.; Filimonov, V.; Nielsen, J. E.; Kursula, I.; Norledge, B. V.; Wierenga, R. K. *Eur. J. Biochem.* **2000**, *267*, 2516–2524; (e) Alvarez, M.; Wouters, J.; Maes, D.; Mainfroid, V.; Rentier-Delrue, F.; Depiereux, E.; Martial, J. A. *J. Biol. Chem.* **1999**, *274*, 19181–19187; (f) Mande, S. C.; Mainfroid, V.; Kalk, K. H.; Goraj, K.; Martial, J. A.; Hol, W. G. *Protein Sci.* **1994**, *3*, 810–821.
3. Hartman, F. C. *Biochemistry* **1971**, *10*, 146–154.
4. Schray, K. J.; O'Connell, E. L.; Rose, I. A. *J. Biol. Chem.* **1973**, *248*, 2214–2218.
5. (a) Collins, K. D. *J. Biol. Chem.* **1974**, *249*, 136–142; (b) Lewis, D. J.; Lowe, G. *J. Chem. Soc., Chem. Commun.* **1973**, 713–715.
6. Wolfenden, R. N. *Nature* **1969**, *223*, 704–707.

7. Lolis, E.; Petsko, G. A. *Ann. Rev. Biochem.* **1990**, *59*, 597–630.
8. Kuntz, D. A.; Osowski, R.; Schudok, M.; Wierenga, R. K.; Müller, K.; Kessler, H.; Opperdoes, F. R. *Eur. J. Biochem.* **1992**, *207*, 441–447.
9. Velanker, S. S.; Ray, S. S.; Gokhale, R. S.; Suma, S.; Balaram, H.; Balaram, P.; Murthy, M. R. N. *Structure (London)* **1997**, *5*, 751–761.
10. Joubert, F.; Neitz, A. W.; Louw, A. I. *Proteins* **2001**, *45*, 136–143.
11. Gomez-Puyou, A.; Saavedra-Lira, E.; Becker, I.; Zubillaga, R. A.; Rojo-Dominguez, A.; Perez-Montfort, R. *Chem. Biol.* **1995**, *2*, 847–855.
12. (a) Tellez-Valencia, A.; Avila-Rios, S.; Perz-Montfort, R.; Rodriguez-Romero, A.; Tuena de Gomez-Puyou, M.; Lopez-Calahorra, F.; Gomez-Puyou, A. *Biochem. Biophys. Res. Commun.* **2002**, *295*, 958–963; (b) Willson, M.; Callens, M.; Kuntz, D. A.; Perie, J.; Opperdoes, F. R. *Mol. Biochem. Parasitol.* **1993**, *59*, 201–210; (c) Espinoza-Fonseca, L. M.; Trujillo-Ferrara, J. G. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 3151–3154.
13. Fonvielle, M.; Weber, P.; Dabkowska, K.; Therisod, M. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 2923–2926.
14. Eubanks, L. M.; Poulter, C. D. *Biochemistry* **2003**, *42*, 1140–1149.
15. Synthesis of **1**–**3b**: 7.82 mmol of either methyl acrylate (→ **1**, **2b**) or acrylonitrile (→ **3b**) were added dropwise to a mixture of 5 mL of diethylphosphite and 0.78 mmol of DBU. After 16 h, the excess of diethylphosphite was evaporated, and the adduct was isolated through a bubble to bubble distillation under reduced pressure (yield 70–90%). The phosphonate esters were cleaved by an excess of TMS–Br. After evaporation, the residue was treated with either a large excess of aqueous hydroxylamine (→ **1b**, **3b**) or 5 molar equivalents of aqueous hydrazine (→ **2b**). After 1 h, the reaction medium was thoroughly evaporated, and the product finally purified as the bis-cyclohexylammonium salt, recrystallized from hot ethanol.

    Selected analytical data of new compounds:

    Compound **2b**: ^{1}H NMR (D$_2$0): δ 1.5 (2H, m); δ 2.15 (2H, m) (δ 2.9: 2H, m; δ 1.3–1.9: 10H, m; δ 0.8–1.3: 10H, m: CHA); ^{13}C NMR (D$_2$0): δ 174.5, 28.3, 22.9 (50.2, 24.2, 30.2, 24.2, 23.7: CHA); ^{31}P NMR (D$_2$0): δ 22.7, s; MS (electrospray, M^{-}): M + 1 = 168 g/mol.

    Compound **3b**: ^{1}H NMR (D$_2$0): δ 1.44 (2H, m); δ 2.17 (2H, m) (δ 2.9: 2H, m; δ 1.3–1.9: 10H, m; δ 0.8–1.3: 10H, m: CHA); ^{13}C NMR (D$_2$0): δ 158.3, 27.5, 25.4 (50.2, 30.3, 25.1, 24.2, 23.7); ^{31}P NMR (D$_2$0): δ 19.5, s; MS (electrospray, M^{-}): M + 1 = 167 g/mol.
16. Weber, P.; Fonvielle, M.; Therisod, M. *Tetrahedron Lett.* **2003**, *44*, 9047–9049.
17. Synthesis of **3a**: 3 mmol of anhydrous glycolonitrile (obtained by successive azeotropic evaporations with pyridine of an aqueous solution, Fluka) and 4.5 mmol of di-*tert*-butylphosphochloridate were dissolved in 10 mL of anhydrous DMF under argon at 0 °C. NaH (9 mmol of 60%) was added portionwise. After stirring 2 h, the mixture was filtered, DMF was evaporated, the residue was redissolved in dichloromethane and washed twice with water. Di-*tert*-butylphosphoglycolonitrile was purified by flash-chromatography (ethyl acetate/pentane 3:7; yield: 61%) and then deprotected by action of dry Amberlite 15 in acetonitrile at reflux for 2 h. After evaporation, the crude phosphoglycolonitrile was treated in water with a 10 equiv excess of aqueous hydroxylamine (from Fluka). After thorough evaporation, the residue was redissolved in aqueous cyclohexylamine and the medium evaporated to dryness. The white crystals of bis-cyclohexylamonium salt **3a** were recrystallized in hot ethanol.

    Selected analytical data of new compounds:

    Di-tert-butylphosphoglycolonitrile: ^{1}H NMR (D$_2$O): δ 4.6 (2H, d, 10 Hz) δ 1.5 (18H, s); ^{13}C NMR (D$_2$O): δ 114.8, (84.4, 84.3), 50.9, 29.7; ^{31}P NMR (D$_2$0): δ 10.6 (1P, t, 10 Hz).
18. (a) Davenport, R. C.; Bash, P. A.; Seaton, B. A.; Karplus, M.; Petsko, G. A.; Ringe, D. *Biochemistry* **1991**, *30*, 5821–5826; (b) Alagona, G.; Ghio, C.; Kollman, P. A. *J. Am. Chem. Soc.* **1995**, 9855–9862; (c) Aqvist, J.; Fothergill, M. *J. Biol. Chem.* **1996**, *271*, 10010–10016.
19. Enzymatic assays: NADH (0.117 mM), glycerol-3-phosphate dehydrogenase from rabbit muscle (Fluka) (1140 mU) and TIM from rabbit muscle (Sigma) (20 mU) were mixed in 25 mM triethanolamine/HCl buffer pH 7.9 (1.5 mL final volume). The cuvet was incubated 10 min at 25 °C. The reaction was started by addition of the substrate G3P (0–250 μM).