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Bioorganic & Medicinal Chemistry Letters 14 (2004) 2923–2926
Bioorganic & Medicinal Chemistry Letters

New highly selective inhibitors of class II fructose-1,6-bisphosphate aldolases

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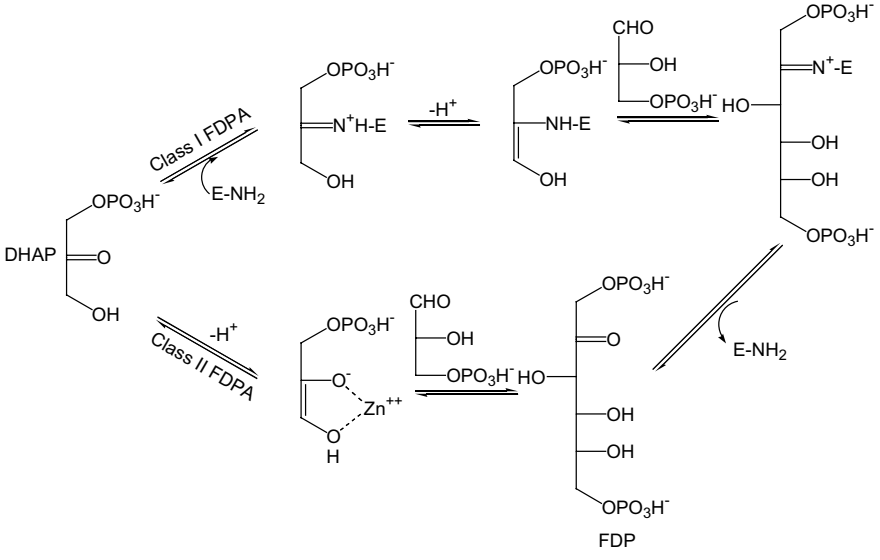
Received 9 January 2004; revised 10 March 2004; accepted 12 March 2004

Abstract—Phosphoglycolo amidoxime and phosphoglycolo hydrazide, two new derivatives of phosphoglycolic acid, were synthesised and successfully tested as selective competitive inhibitors of class II FBP-aldolases.

Fructose bisphosphate aldolase (EC 4.1.2.13) catalyses the reversible cleavage of fructose-1,6-bisphosphate (FBP-aldolase) into dihydroxyacetone-phosphate and glyceraldehyde-3-phosphate. Two main classes of this enzyme are present in living organisms, which strongly differ in their structures.[1,2]

Class I aldolase is widespread in all kingdoms (plants, bacteria, animals…), while class II is only found in micro-organisms (bacteria, yeasts). Although the two classes catalyse the same reaction with the same stereospecificity, they act through very different mechanisms (Scheme 1). With class I aldolase, an enamine



Scheme 1. Mechanism of the formation/cleavage of FBP catalysed by FBP-aldolase class I and class II.

Keywords: Fructose bisphosphate aldolase; Inhibitor; Hydrazide; Amidoxime; Metalloenzyme.
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doi:10.1016/j.bmcl.2004.03.040

(imminium) is formed between the ketose and a lysine residue of the enzyme, which will act as a donor (acceptor) for the formation (cleavage) of the C–C bond.

With class II aldolase, a «true» enolate is formed, stabilised by a Zn^{++} ion within the active site. The tertiary structure of a class II FBP-aldolase (from *Escherichia coli*) has recently been published,[2] giving a rather unambiguous role of Lewis acid to the zinc ion present into the active site.

Class II aldolase is present in many pathogenic bacteria and yeasts, like *Helicobacter pylori*, *Mycobacterium tuberculosis*, *M. pneumoniae*, *M. leprae*, *Yersinia pestis*, *Candida albicans*, while class I is the only form present in mammals.

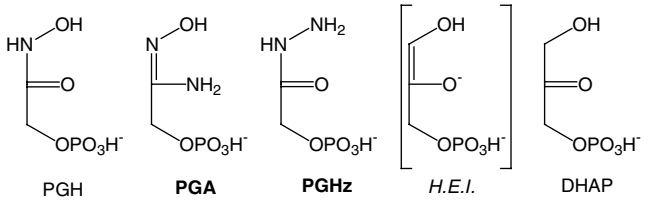
In *E. coli*, mutations of the gene coding for FBP-aldolase have been shown to severely disturb the metabolism or slow down the growth of the bacteria.[3,4] Attempts to prepare knockout mutants of *Streptomyces* have failed, indicating that an absence of the (class II) FBP-aldolase might be lethal in these species.[5] Consequently, good selective inhibitors of class II FDP-aldolase are potential synthetic antibiotics of interest (in fact, this was pointed out as early as 1973 by Lewis in a key publication[6]). However, making these hydrophilic compounds able to cross bacterial membranes will be another challenge.

Very surprisingly, while tens of inhibitors of class I have been synthesised and tested,[7] only one good inhibitor of class II is known: phosphoglycolo hydroxamic acid (PGH). Syntheses of this compound has been reported in two concomitant papers by Lewis and Lowe and Collins.[6,8] PGH is a remarkable competitive inhibitor of yeast class II aldolase, with a K_i of 10 nM ($K_M/K_i = 40,000$). The compound is much less active on mammalian aldolase (class I), with a K_i of 1 μM. Thus, the selectivity of PGH for class II FDP-aldolase is 100 (based on K_i values).[8] As demonstrated later by X-ray analysis of a class II aldolase crystallised in the presence of PGH,[2] this compound is both a zinc chelator and a «transition-state analogue» of the reaction (precisely, a stable analogue of the intermediate enediolate).

We reasoned that other functional groups born by the phosphoglycolate skeleton could have similar properties, thus permitting to enlarge this rather limited family (one member!) of inhibitors. Hydroxamic acid is a well known chelating group of Zn^{++}, and this property has been exploited in the synthesis of numerous inhibitors of metalloenzymes, mostly zinc-hydrolases.[9] Other nitrogen-based chelators include amidoximes[10] and hydrazides.[11]

However, while hydrazides have been tested with some success as enzymes inhibitors,[12] there are only few examples of the use of amidoximes.[13]

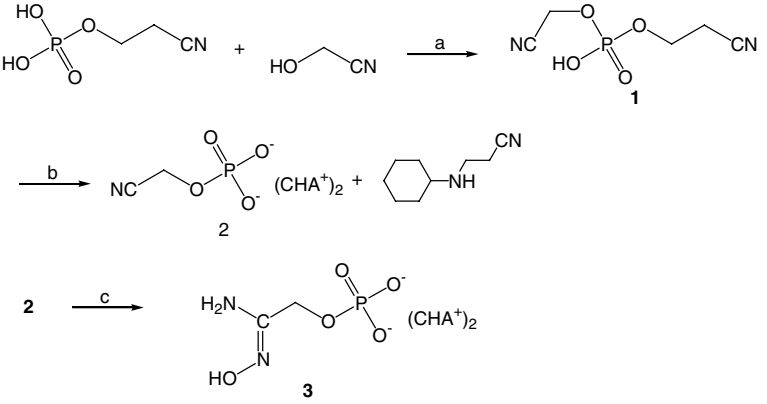
We prepared phosphoglycolo-amidoxime (PGA) and phosphoglycolo-hydrazide (PGHz), two products, which have not been described in the literature, in spite of their very simple structures (Scheme 2).



Scheme 2. Structures of phosphoglycolic acid derivatives: hydroxamic acid (PGH), amidoxime (PGA), hydrazide (PGHz); DHAP: substrate of FDP-aldolase; H.E.I.: enediolate, the high energy intermediate formed by deprotonation of DHAP.

As shown in Scheme 2, these compounds are targeted as (at least) analogues of the substrate DHAP, or (at best) analogues of the intermediate enediolate and as zinc-chelators. PGHz was synthesised in two steps from glycoloamide as reported previously.[14] PGA was prepared from glycolonitrile according to Scheme 3.[15]

The deprotection step (b), usually performed with aqueous sodium hydroxide, was simplified by the use of cyclohexylamine, with the following advantages: (1) direct formation of the bis-CHA salt of the phosphate,



Scheme 3. Synthesis of PGA **3**. Reagents and conditions: (a) DCC, Py, 60°, 24 h, then 96 h rt; (b) cyclohexylamine 1 M/H_2O, 4 h 0 °C, then 1 h rt; (c) NH_2OH/H_2O, rt, 6 h.

readily purified by crystallisation from ethanol; (2) trapping of the by-product acrylonitrile as a CHA adduct, thus avoiding formation of tedious polymers. PGA and PGHz were tested as inhibitors of two class II aldolases (from yeast and from *Bacillus subtilis*) and of a class I aldolase (from rabbit muscle).[16] PGA behaved clearly as a competitive inhibitor of both class II aldolases, in a test using FBP as a substrate (Fig. 1).

By comparison, no inhibition was measured on rabbit muscle aldolase at a concentration up to 1 mM.

PGHz was shown similarly to be a competitive inhibitor of the yeast (Fig. 2) and *B. subtilis* enzymes.

The inhibition of rabbit muscle aldolase was again much weaker, apparently of a mixed type (Fig. 3).

Thus, both tested compounds show respectable performance as competitive inhibitors of class II aldolases. However, they are by two orders of magnitude less effective than the old known PGH (Table 1).

This suggests that PGA and PGHz act as analogues of the substrate dihydroxyacetone-phosphate rather than analogues of the enediolate intermediate, as does PGH. In addition and as expected, they must be good chelators of the zinc ion present at the active site, as indicated by the large difference observed in inhibitions of class I and class II.

In spite of their weaker activity as compared to PGH, the two new inhibitors show a better selectivity for the class II aldolase than PGH[8] (Table 2).

From this point of view, they might be better candidates as potential drugs than the too efficient PGH. Moreover, PGA and PGHz seem more stable in (frozen) aqueous solution than PGH: in our hands, frozen solutions of this later compound lost rapidly their inhibition power,

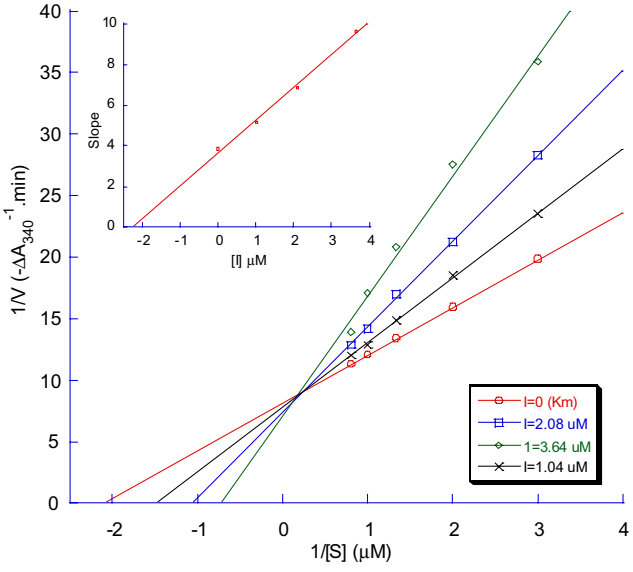


Figure 1. Inhibition of FBP-aldolase class II from yeast by PGA.

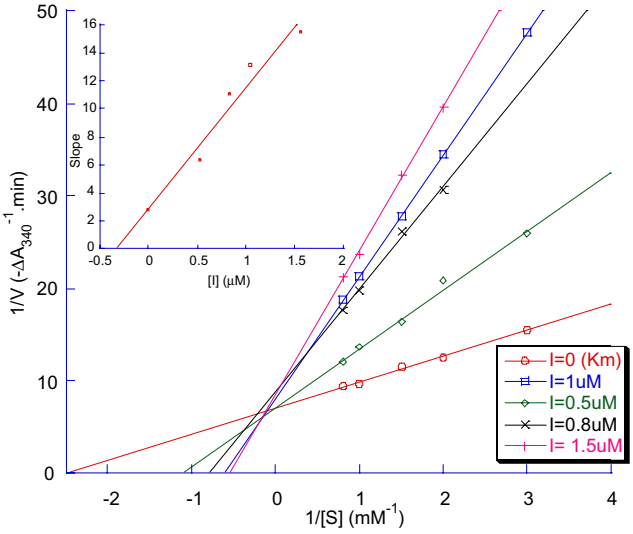


Figure 2. Inhibition of FBP-aldolase class II from yeast by PGHz.

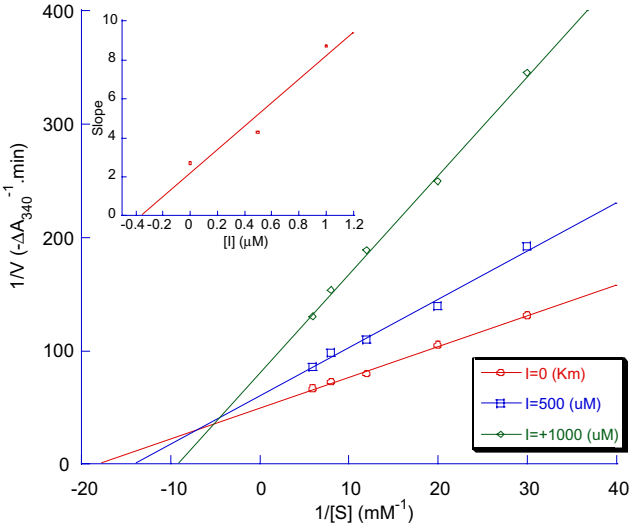


Figure 3. Inhibition of FBP-aldolase class I from rabbit by PGHz.

Table 1. Enzymatic kinetics constants (μM) measured on substrates/inhibitors

Aldolase source	K_m	K_i		
	FBP	PGA[a]	PGHz[a]	PGH
Rabbit	55	>1000	370	1
B. subtilis	2000	3.3	0.67	—
Yeast	450	2.3	0.34	0.01

[a] As bis-cyclohexylammonium (CHA) salts; CHA chloride was previously shown to have a K_i on aldolases >6 mM.[8]

Table 2. Selectivity of inhibitors for class II versus class I FBP-aldolase

	PGA	PGHz	PGH
$K_{i_{rabbit}}/K_{i_{yeast}}$	>435	1088	100
$K_{i_{rabbit}}/K_{i_{B.subtilis}}$	>303	552	—

while solutions of PGA and PGHz, stored in similar conditions, remained active for weeks.

So, we think that PGHz and PGA could contribute to the development of a new class of antimicrobial drugs.

Acknowledgements

We are grateful to Celine Roux for her help and helpful discussions during the enzymatic tests.

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15. Synthesis of PGA (**3**): A suspension of baryum β-cyanoethylphosphate (30 mmol) in water was shaken with Dowex 50 (H^+) until all organic material dissolved. The aqueous solution was evaporated to dryness, and the residue dried by coevaporation with pyridine, toluene and finally dissolved in 30 mL of anhydrous pyridine. 2.5 mL (25 mmol) of a commercial solution of glycolonitrile in water (55% w/w, Fluka) was similarly coevaporated in the presence of pyridine, toluene and the syrupy residue redissolved in the previous solution of β-cyanoethylphosphate in pyridine. 100 mmol of DCC were added. The mixture was stirred 24 h at 60 °C under argon and four more days at rt. 5 mL of water were added to the black mixture, and after 1 h the whole reaction medium was evaporated to dryness. The residue was suspended in 200 mL water, and the insoluble DCU filtered. The filtrate was evaporated, and the black residue extracted 3 times with 100 mL of boiling dichloromethane. The combined extracts were evaporated, giving 3 g of crude **1** (and unreacted glycolonitrile).
The syrup was redissolved in 90 mL of 1 M cyclohexylamine in water and left 4 h at 0 °C and 1 h at rt. After evaporation, the residue was extracted twice with dichloromethane, redissolved in water and the aqueous solution again extracted twice with DCM. The aqueous phase was finally evaporated to give 2.1 g (25%) of crude dicyclohexylammonium salt of phosphoglycolonitrile (**2**), recrystallised from hot ethanol.
Compound **2** was quantitatively converted to the amidoxyme **3** by treatment with 2 equiv of aqueous hydroxylamine at rt during 6 h. After complete evaporation of the reaction medium, **3** was recrystallised from hot ethanol.
Selected analytical data:
Compound **2**: ^{1}H NMR (D_2O) δ 4.3 (2H, d, 10 Hz) (δ 2.9: 2H, m; δ 1.3–1.9: 10H, m; δ 0.8–1.3, 10H, m: CHA).
^{13}C NMR (D_2O) δ 119.00, 119.08, 61.60, 61.65 (51.1, 31.2, 25.2, 24.7: CHA).
Compound **3**: ^{1}H NMR (D_2O) δ 4.0 (2H, d, 6 Hz) (δ 2.9: 2H, m; δ 1.3–1.9: 10H, m; δ 0.8–1.3, 10H, m: CHA).
^{13}C NMR (D_2O) δ 156.1, 156.2, 61.58, 61.54 (51.1, 31.2, 25.16, 24.7: CHA).
HRMS (EI, M^+) (tris-trimethylsilyl derivative): calcd for $C_{11}H_{31}N_2O_5PSi_2$: 386.1278; found: 386.1272.
16. Enzymes: Rabbit muscle and *B. subtilis* FDP-aldolases (Fluka) had specific activities of 13 and 30 U/g, respectively. Aldolase from yeast was partly purified according to Ref. 17 after disruption of the cells in a French press. Specific activity: 5.5 U/mg.
Enzymatic assays: DHAP formed by cleavage of FBP by aldolase was estimated by measuring spectrophotometrically (340 nm) the consumption of NADH in a coupled system employing a 300-fold excess of glycerophosphate dehydrogenase (GDH). Incubation medium: Class I aldolase: triethanolamine–HCl buffer pH 7.6, 0.1 M, EDTA 1 mM; Class II aldolase: glycylglycine buffer pH 7.4, 0.1 M, 2-mercaptoethanol 0.01 M (omitted for *B. subtilis* aldolase).
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