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New competitive inhibitors of cytosolic (NADH-dependent) rabbit muscle glycerophosphate dehydrogenase

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Abstract—We report the synthesis and biochemical evaluation of new competitive inhibitors of the cytosolic (NADH-dependent) glycerophosphate dehydrogenase. The best tested compound, phosphono-propionohydroxamic acid, with a K_i of 6 μM, might be of interest as an anti-obesity drug.

Cytosolic glycerophosphate dehydrogenase (GPDH; EC 1.1.1.8) has an established role in forming the glycerol backbone of triglycerides by catalyzing the biosynthesis of glycerol-3-phosphate within the glycolysis pathway.[1] Thus, GPDH contained in adipocytes is a key enzyme for the metabolic conversion of glucose to triglycerides. It is strongly involved in storage of fat originating from absorbed carbohydrates, eventually leading in some cases to obesity. Considerable efforts are being made to control the biosynthesis of triglycerides and reduce the incidence of this disease in developed countries. Several reports indicate that the level of GPDH activity correlates well with the cellular transformation into adipose cells,[2] and even with an inhibition of lipogenesis.[3] Substances shown to reduce the cellular activity of GPDH can act either by direct inhibition of the enzyme or by lowering its expression within the cell. Some natural products like vitamin C and (−)-catechin (lipolytic constituents of green-tea[3]), or pycnogenol,[4] a mixture of vegetal flavonoids extracted from various sources are active through this second mechanism. Human growth hormone has the same effect on cultivated rat adipocyte precursor cells.[5] Polyphenols extracted from *Salacia reticulata*, a plant known for its anti-obesity action, directly inhibit GPDH.[6] Alkenylresorcinols extracted from wheat or rye bran also are efficient inhibitors (IC_{50} 4–6.5 μM) of the enzyme.[7] Several patents describe and protect the utilization of GPDH inhibitors present in food additives as anti-obesity agents.[8] Simple substances like inorganic phosphonate,[9] alkylammonium chloride,[10] sodium octanoate,[11] cinnamic acid derivatives[12] or inorganic anions[13] are also weak or moderate inhibitors of GPDH. The enzyme was also shown to be inactivated by non-specific inhibitors like *N*-alkylmaleimide[14] and *p*-chloromercurybenzoate.[15] On the other hand, several anti-parasitic drugs (e.g., cymelarsan, suramin, LG1, active against *Leishmania* or *Trypanosoma*) have been shown to selectively inhibit the parasite GPDH, with remarkable IC_{50} values in the micromolar range, versus 100–1000 μM against the rabbit enzyme.[16] Phosphoglycolohydroxamate (PGH) was synthesized 30 years ago[17,18] and tested against a variety of DHAP utilizing enzymes.[19] It is a kind of universal inhibitor, however with only little selectivity. This compound inhibits rabbit-muscle PGH with a K_i of 10 μM.[18]

Thus, a survey of literature shows a need for specific synthetic inhibitors of mammalian GPDH.

We previously reported the synthesis and evaluation of several potential inhibitors of fructose-bisphosphate aldolases (FBA) and of triose phosphate isomerase (TIM) (**1**–**3**, Fig. 1).[20,21]

We reasoned that these compounds could also be inhibitors of other enzymes acting on DHAP, and we here report the results of the inhibition tests of this series on the cytoplasmic glycerophosphate dehydrogenase (GPDH) from rabbit muscle. We consider this commercial enzyme as a good model of human GPDH, with which it shares 92% sequence identity.

Keywords: Anti-obesity drugs; Triglycerides metabolism; Enzymes inhibitors; Organic phosphonates; Hydroxamic acids; Glycerophosphate dehydrogenase.
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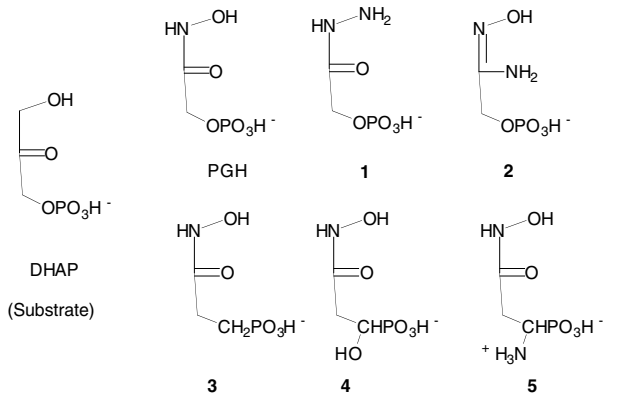


Figure 1. Potential synthetic inhibitors of enzymes utilizing DHAP as a substrate.

To complete the family of these potential inhibitors, we also prepared compounds **4** and **5**. In our mind, all these products were supposed to act as structural analogues of the substrate DHAP. In compounds **1** and **2**, the hydrolysable hydroxamic acid function of PGH was replaced by more stable hydrazide and amidoxime. Since phosphate groups can readily be cleaved by phosphatases under physiological conditions, the phosphate group of PGH was tentatively replaced in **3** by a methylene phosphonate.

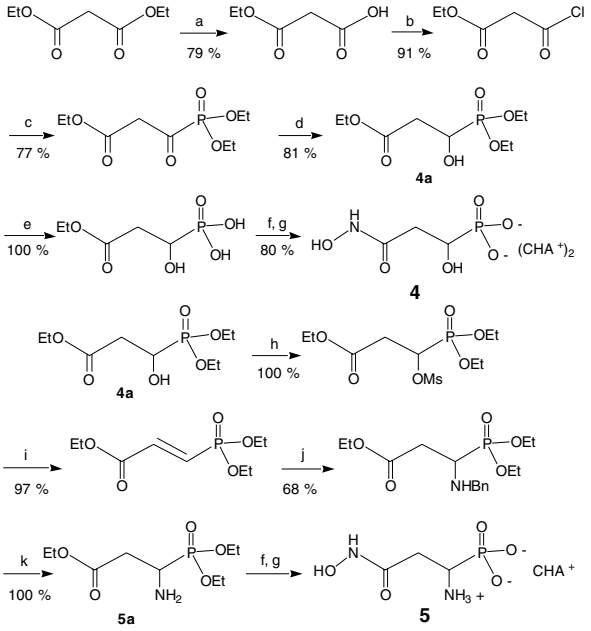
The phosphoester oxygen of DHAP might be acceptor of a hydrogen bond with a residue of the active site of the enzyme. This interaction cannot exist with **3**. We prepared compound **4** with the hope to restore this hypothetic hydrogen bond. Also, phosphonates and phosphates have significantly different p*K*a (Table 1), and consequently their ionisation states can be different at a given pH (for example at pH 7, DHAP should be a di-anion and **3** a mono-anion). To have a phosphonate with a p*K*a of a phosphate, we synthesized compound **5**.

The preparation of compounds **1**–**3**,[20,21] together with an improved synthesis of PGH[23] was reported previously. Compounds **4**[24], and **5**[25] were prepared according to Scheme 1.

All the tested compounds gave purely competitive inhibitions in a test using DHAP and NADH as substrates of GPDH.[26] The results are reported in Table 2. We consider products with $K_i > 0.5$ mM as ineffective inhibitors (**1** and **5**), and in these cases, the K_i values were not determined accurately. Compounds **2** and **4** showed intermediate performances, with K_i around 100 and 40 μM, respectively. Phosphonopropionohydroxamate (**3**) is the most interesting product of this series, with a

Table 1. Typical p*K*a values of various phosphates and phosphonates[22]

Compound	pK_a
DHAP	1.8/6.45
CH_3–$(CH_2)_3$–$PO(OH)_2$	2.6/8.2
$^+NH_3$–CH_2–$PO(OH)_2$	1.85–2.35/5.35–5.9



Scheme 1. Syntheses of (racemic) 3-hydroxy-3-phosphono-propionohydroxamic acid **4** and 3-amino-3-phosphono-propionohydroxamic acid **5**. Reagents: (a) KOH (1 M equiv); (b) $SOCl_2$; (c) $P(OEt)_3$; (d) Pyr–borane/HCl; (e) TMSBr; (f) NH_2OH/H_2O; (g) cyclohexylamine; (h) MsCl; (i) NEt_3; (j) $BnNH_2$; (k) H_2/Pd.

Table 2. Inhibition[a] of DHAP utilizing enzymes by compounds **1**–**5**

Compound	Class I FBA	Class II FBA	TIM	GPDH[c]
PGH[b]	1	0.01	3	10
1	370	**0.34**	111	>500
2	>1000	2.3	**4.5**	100
3	>500	145	160	**6**
4	180	418	>1000	40
5	9000[d]	3000[d]	>500	>500

[a] K_i, (μM).
[b] K_i values determined in this work; in accordance with those reported in Refs. 17 and 18.
[c] K_M (DHAP) = 125 mM.
[d] Calculated from IC_{50} values.

K_i of 6 μM. This is one rare example of a phosphonate being at least as active as the corresponding phosphate (PGH). The good inhibition of GPDH by **3** is a strong indication of the absence of hydrogen bond between a residue of the active site of GPDH and the phosphoester oxygen of its substrate. The hydroxyl and ammonium groups introduced in **4** and **5**, respectively, do not have the expected beneficial effect. On the contrary, inhibition of GPDH follows the order **3** > **4** > **5** and correlates with the bulkiness of the substituent alpha to phosphorus (H in **3**, OH in **4**, NH_3^+ in **5**). Compounds **4** and **5** were also disappointing inhibitors of PGA and TIM.

The K_i and K_M/K_i values (6 μM and 21, respectively, for **3**) are consistent with this inhibitor acting as a substrate analogue (of DHAP) rather than as a transition-state or a high-energy intermediate analogue. An overall summary of the properties of compounds **1**–**5** on GPDH and other glycolytic enzymes is presented in Table 2.

In conclusion, we have devised the synthesis and evaluation of new competitive inhibitors of mammalian cytosolic glycerophosphate dehydrogenase. These products (or their prodrug derivatives) may have applications as anti-obesity drugs. One of these compounds, phosphonopropionohydroxamate **3**, is of special interest for the following reasons:

— Being a phosphonate, it is insensitive to hydrolysis by phosphatases.
— It does not interfere with other enzymes of the glycolysis, namely FBP-aldolase and triosephosphate isomerase, for which it is an only very poor inhibitor.

Compounds **3** and **4** could also be of interest for further studies of the catalytic mechanism of GPDH based on structural analyses (the crystal structure of the human enzyme was reported recently.)[27]

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2006.10.030.

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24. Synthesis of **4**: 47 mmol of triethyl phosphite were added dropwise to a solution of ethylmalonyl chloride in diethylether at 0 °C. After 72 h at room temperature, the excess of triethyl phosphite was evaporated, and the product (for a large part in enol form, as deduced from its ^{1}H NMR spectrum) distilled (97 °C/0.17 mm). It was then treated by 1.5 M equivalent of pyridine–borane in presence of HCl. After neutralization and evaporation of the mixture, the protected hydroxyphosphonate **4a** was dissolved in dichloromethane, washed with water, and purified by flash chromatography (AcOEt/MeOH/H_2O, 90:10:1). The final product was obtained after deprotection with pure TMS–Br (2 h, rt), evaporation, and treatment with an excess of aqueous hydroxylamine. Compound **4** was purified by crystallization of the biscyclohexylammonium salt in methanol/AcOEt.
Compound **4a**: ^{1}H NMR ($CdCl_3$): δ 1.24 (9H, m); 2.66 (2H, m); 4.12 (6H, m); 4.36 (1H, m); 6.72 (1H, br s).
^{13}C NMR ($CdCl_3$, BB): δ 13.6; 15.9; 36.4; 62.4; 62.8; 60.5; 60.7; 65; 65.1; 170.3.
^{31}P NMR ($CdCl_3$, BB): δ 24.4.
ESI HR-MS: 277.0818 g/mol (M+Na); Th: 277.0811 g/mol.
Compound **4**: ^{1}HNMR (D_2O): δ 0.75–1.05 (10H, m); 1.25–1.64 (10H, m); 2 (1H, ddd; 15, 12, 8 Hz); 2.2 (1H, ddd; 15, 4, 3 Hz); 2.79 (2H, m); 3.6 (3H, ddd; 12, 8, 3 Hz).
^{13}C NMR (D_2O, BB): δ 23.6; 24.3; 29.9; 36.5; 50.1; 65.9; 67.6; 171.
^{31}P NMR (D_2O, BB): δ 15.8.
ESI HR-MS: 184.0009 g/mol (M+1); Th: 184.0005 g/mol.
25. Synthesis of **5**: the intermediate triethyl ester **4a** was mesylated by a standard method (MsCl/NEt_3 in refluxing dichloromethane) and treated by another equivalent of NEt_3 to eliminate the OMs group. Michael addition of benzylamine was performed using 2 equiv of base in refluxing ethanol, overnight, followed by flash chromatography (pentane/AcOEt, 3:7). The amine was debenzylated by hydrogenolysis (Pd/C, 2 atm) in ethanol to give **5 a**. Deprotection of the phosphate, hydroxylaminolysis of the ester and isolation of **5** as the cyclohexylammonium salt were performed as for **4**.
Compound **5a**: ^{1}HNMR (D_2O): δ 1.28 (3H, t, 7 Hz); 1.35 (6H, t, 7 Hz); 2 (2H, br s); 2.49 (1H, ddd; 16, 11, 8 Hz); 2.8 (1H, ddd; 16, 8, 3 Hz); 3.17 (1H, ddd; 16, 11, 3 Hz); 3.68 (6H, q, 7 Hz).
^{13}C NMR (D_2O, BB): δ 14; 16.3; 16.4; 36.8; 44.3; 46.7; 60.7; 62.2; 62.3; 62.4; 170.9; 171.2.
ESI HR-MS: 276.0975 g/mol (M+Na); Th: 276.0971 g/mol.
Compound **5**: ^{1}H NMR (D_2O): δ 0.89–1.71 (10H, m);

2.38 (1H, ddd; 16, 11, 7 Hz); 2.51 (1H, ddd; 16, 7, 3 Hz); 2.88 (1H, m); 3.14 (1H, ddd; 12, 11, 3 Hz).
^{13}C NMR (D_2O, BB): δ 23.7; 24.2; 30.3; 31.2; 46; 48.1; 50.2; 169.5.
^{31}P NMR (D_2O, BB): δ 10.2.
ESI MS: 367 g/mol (2M+1).

26. Enzymatic assays: NADH (0.2 mM), dihydroxyacetone phosphate and the inhibitor were dissolved in a 50-mM Tris–HCl buffer, pH 7.4 (final volume: 1 mL), at 25 °C. The reaction was started by addition of the enzyme (GPDH from rabbit muscle, Sigma) and the UV absorbance at 340 nm was monitored on a spectrophotometer as a function of time.
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