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Separate synthesis and evaluation of glucitol bis-phosphate and mannitol bis-phosphate, as competitive inhibitors of fructose bis-phosphate aldolases

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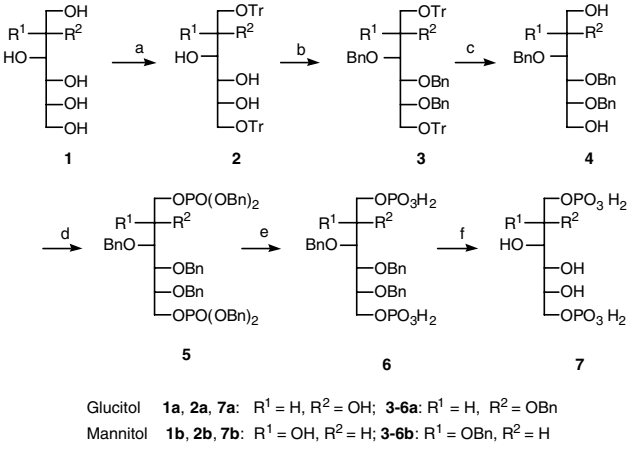
Abstract—We report the first unambiguous syntheses of glucitol-1,6-bis-phosphate and mannitol-1,6-bis-phosphate and their competitive inhibition of various fructose bis-phosphate aldolases.

Hexitol bis-phosphate (HBP), a diastereoisomeric mixture of mannitol bis-phosphate and glucitol bis-phosphate, is known to inhibit or activate several enzymes. This mixture was first tested on class I muscle fructose bis-phosphate aldolase (Fba), for which it is a competitive inhibitor[1,6] ($K_i \sim 1.2$ μM). Later, it was also recognized as an inhibitor of yeast (class II) Fba[2] ($K_i \sim 200$ μM), of pyruvate kinase[3] and of fructose bis-phosphate phosphatase.[4] On an other hand, HBP is an activator of 6-phosphofructo-kinase.[5]

Hexitol bis-phosphate is routinely synthesized according to Ginsburgh by reaction of sodium borohydride on fructose bis-phosphate.[6] We determined by GC of a resultant per-silylated mixture that it was composed of 60% mannitol bis-phosphate and 40% glucitol bis-phosphate.

Surprisingly, the two constituents of this mixture have never been synthesized nor tested separately against glycolytic aldolases, and the kinetic constants reported above are thus only apparent constants. Glucitol bis-phosphate was tentatively prepared by Hartman,[7] however the product was not characterized, and in view of our own observations, there may be doubt as to its identity. Nevertheless, it was reported to be a good competitive inhibitor of liver[8] and of muscle Fba,[7] with $K_i \sim 4.5$ μM and 12 μM, respectively.



Scheme 1. Synthesis of glucitol bis-phosphate **7a** and mannitol bis-phosphate **7b**.[11] Reagents and conditions: (a) TrCl/pyridine RT 48 h; (b) BnBr NaH/DMF RT; (c) TFA/BuOH/CH_2Cl_2 RT 48 h; (d) $^{i}Pr_2NP(OBn)_2$/Imidazole/Triazole/AcCN RT 48 h, then $^{t}BuOOH$; (e) H_2 (1 bar)/Pd–C/NEt_3, then Dowex 50 (H^+); (f) H_2 (1 bar)/Pd–C.

Keywords: Aldolase; Fructose-bis-phosphate; Enzymes inhibitors; Glycolysis; Mannitol-bis-phosphate; Glucitol-bis-phosphate; Hexitol-bis-phosphate.
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The synthesis of mannitol bis-phosphate has simply never been reported. This last compound, however, is of special interest, especially regarding its action on Fba: It was recently reported that by soaking crystals of Fba with a solution of HBP, only mannitol bis-phosphate was retained in the active site of the enzyme.[9]

We report hereby the synthesis and separate testing of glucitol and mannitol bis-phosphate on class I and class II Fba (EC 4.1.2.13).

Although the synthesis (Scheme 1) appears straightforward, successful yield of a pure product is strongly dependant on particular attention paid during final deprotection steps. It is well known that a protected phosphoryl group adjacent to a free hydroxyl can readily migrate to this free hydroxyl.[10] In polyols, the result is a complex mixture of regioisomers. This scrambling can be avoided if the phosphoryl groups are deprotected prior to the hydroxyls. Thus, we used a two-step deprotection protocol: the phosphoryl groups were first debenzylated in presence of triethylamine. In these conditions, the rate of hydrogenolysis of a benzyl ether is apparently considerably reduced. After removal of the tertiary amine on an acidic ion-exchange resin, the benzyl protecting-groups of the hydroxyls were removed classically from the acidic intermediate. The two compounds were subsequently crystallized and characterized as their cyclohexylammonium salts.

Glucitol bis-phosphate and mannitol bis-phosphate were each tested for their inhibition properties against

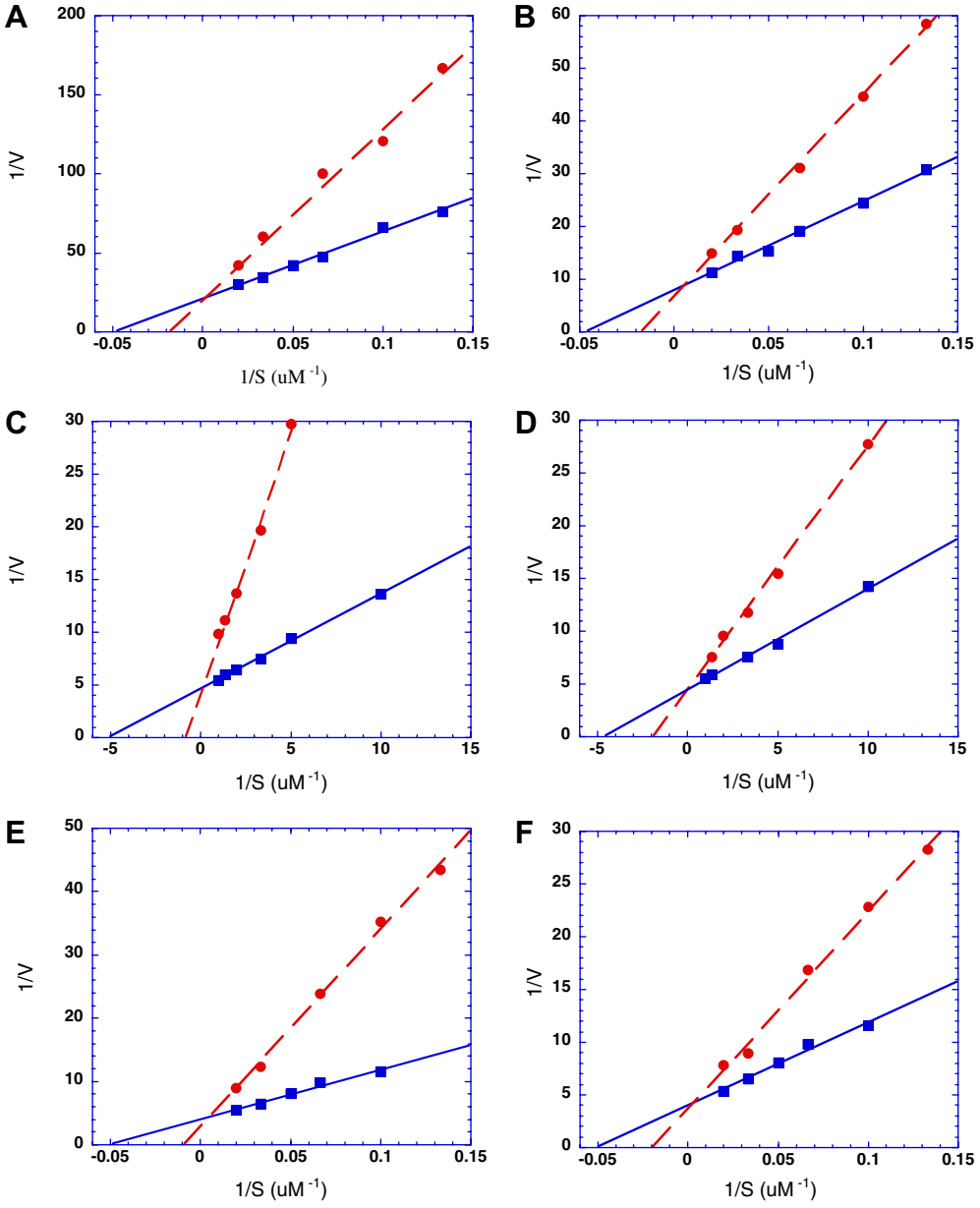

Figure 1. Inhibition of Fba by glucitol and mannitol bis-phosphate. (A) Rabbit muscle Fba, **7a** 0.15 mM; (B) Rabbit muscle Fba, **7b** 0.01 mM. (C) Yeast Fba, **7a** 0.3 mM; (D) Yeast Fba, **7b** 0.6 mM; (E) *H. pylori* Fba, **7a** 0.5 mM; (F) *H. pylori* Fba, **7b** 0.1 mM.

Table 1. Enzymatic kinetics constants (μM) measured on substrates/inhibitors[13]

Aldolase source	K_M (FBP)	K_i (K_M/K_i)	
		7a	**7b**
Rabbit muscle	20	100 (0.2)	7.3 (2.74)
Yeast	200	60 (3.33)	400 (0.5)
H. pylori	20	170 (0.12)	73 (0.27)

rabbit muscle aldolase (representative of class I aldolases), yeast and *Helicobacter pylori* aldolases (representative of class II aldolases), using established protocols based on use of fructose bis-phosphate (FBP) as a substrate.[12] The two compounds displayed purely competitive inhibition patterns against the three enzymes (Fig. 1). The measured kinetic constants are reported in Table 1.

The data shown in Table 1 shows that mannitol bis-phosphate **7b** is a better inhibitor of class I aldolase than the glucitol epimer **7a**. This observation is in full accordance with the structural results reported by St-Jean et al.[9]

For class II aldolases, no trend is readily discernable with regard to preferential inhibition of either enzyme. Compound **7a** is a better inhibitor of the yeast enzyme while **7b** is better against *H. pylori* aldolase. **7a** and **7b** give comparable K_M/K_i values on yeast and rabbit muscle aldolases, respectively. In conclusion, we have reported for the first time separate synthesis of glucitol- and mannitol-1,6-bis-phosphate. The two products have been tested separately as inhibitors of fructose bis-phosphate aldolases from various sources. New inhibitors of these enzymes are of special interest. Fba is active in glycolysis, a major metabolic pathway of virtually all living organisms. Inhibitors of class II Fba can be broad potential drugs against microorganisms.[16] Like other inhibitors of glycolytic enzymes, and depending on their selectivity, inhibitors of class I Fba can also be active against parasites[17] and even cancer.[18] In this perspective, compound **7b** is a promising basis for further syntheses of selective inhibitors of class I aldolases.

Acknowledgments

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Supplementary data

Supporting informations: detailed synthesis of **7a**, **7b**. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.01.076.

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11. Selected analytical data: Glucitol-1,6-bis-phosphate (tetrakis-cyclohexylammonium salt): ^{1}H NMR (D_2O) δ 1.2 (m, 10H) 1.5–1.9 (m, 10H) 3 (m, 2H) 3.6–3.9 (m, 8H). ^{13}C NMR (BB) (D_2O) δ (23.95, 24.5, 30.5, 50.5: CHA) 65.58 (d, J_{C-P}4.7 Hz, C-6 or C-1), 65.8 (d, J_{C-P} 4.7 Hz, C-6 or C-1), 69.7 (s, C-3 or C-4), 70.76 (s, C-3 or C-4), 70.5 (d, J_{C-P} 6.5 Hz, C-2 or C-5), 72.4 (d, J_{C-P} 6.5 Hz, C-2 or C-5). ^{31}P NMR, (BB) (D_2O) δ 2.5, 3.07. $[\alpha]_D^{20}$ − 1.4° (c 2, H_2O) Mannitol-1,6-bis-phosphate (tetrakis-cyclohexylammonium salt): ^{1}H NMR (D_2O) δ 1–1.25 (m, 20H) 1.5–2 (m, 20H)) 3 (m, 4H) 3.6–3.9 (m, 8H). ^{13}C NMR (BB) (D_2O) δ (23.75, 24.25, 30.3, 50.2: CHA) 65.5 (d, J_{C-P} 3.9 Hz, C-1, C-6) 68.4 (C-3, C-4) 70.1 (d, J_{C-P} 6.3 Hz, C-2, C-5). ^{31}P NMR, (BB) (D_2O) δ 4.5. $[\alpha]_D^{20}$ − 3.25° (c 8, H_2O).
12. Enzymes: Rabbit muscle aldolase was from Fluka. Aldolase from baker yeast was partly purified according to Ref. 14 after disruption of the cells in a French press. Aldolase from *H. pylori* is a recombinant enzyme expressed in *E. coli* JM 109. Enzymatic assays: DHAP formed by cleavage of FBP by aldolase was estimated by measuring spectrophotometrically (at 340 nm) the consumption of NADH in a coupled system employing a 300-fold excess of glycerophosphate dehydrogenase and triose phosphate isomerase in glycyl-glycine buffer 0.1 M, pH 7.4, containing 0.2 M potassium acetate.
13. The same buffer system was used for the three enzymes. It should be noted that K_M (K_i) values of rabbit muscle aldolase for its substrate (inhibitor) are influenced by the presence of salts[15] and kinetic parameters can thus vary depending on the salt composition in the assay protocol. This statement assumes that K_M and K_i are affected to the same extent.
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