ACS Medicinal Chemistry Letters

LETTER

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Mannitol Bis-phosphate Based Inhibitors of Fructose 1,6-Bisphosphate Aldolases

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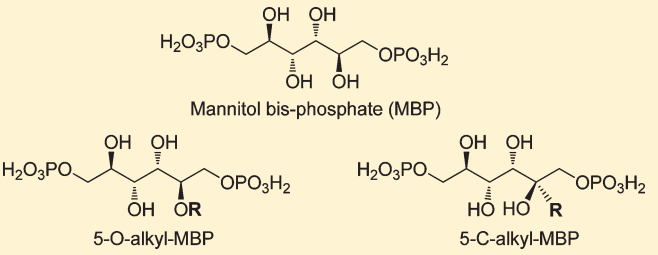
Supporting Information

ABSTRACT: Several 5-*O*-alkyl- and 5-*C*-alkyl-mannitol bis-phosphates were synthesized and comparatively assayed as inhibitors of fructose bis-phosphate aldolases (Fbas) from rabbit muscle (taken as surrogate model of the human enzyme) and from *Trypanosoma brucei*. A limited selectivity was found in several instances. Crystallographic studies confirm that the 5-*O*-methyl derivative binds competitively with substrate and the 5-*O*-methyl moiety penetrating deeper into a shallow hydrophobic pocket at the active site. This observation can lead to the preparation of selective competitive or irreversible inhibitors of the parasite Fba.



KEYWORDS: Selective inhibitors, microbial enzymes, fructose bis-phosphate aldolase, glycolysis, *Trypanosoma brucei*

Parasitic diseases caused by Trypanosomatidae (Chagas' disease, sleeping sickness, leishmaniasis, ...) are members of "neglected diseases" that include tuberculosis and malaria.[1] The two human infective subspecies of *Trypanosoma brucei*, *T. b. gambiense* and *T. b. rhodesiense*, cause 66 000 deaths every year in many sub-Saharan countries, and another 50 million people are at risk of contracting the disease.[2] The social impact of the disease is worsened by massive infection of cattle in these countries by another subspecies, *T. b. brucei*. The parasites reside in the bloodstream of infected patients, and glycolysis represents the only metabolic process through which their ATP is synthesized.[3] This pathway is considered as a promising therapeutic target in the struggle against *T. brucei* infections.[4] Recent reports have shown that only a partial depletion of some glycolytic enzymes leading to the reduction of the glycolytic flux to approximately 50% of the wild-type levels is sufficient to kill the parasites in culture.[5,6] In bloodstream-form *T. brucei*, fructose bis-phosphate aldolase (FBA) has been genetically validated as a drug target by RNA interference. Humans have three aldolase isozymes localized in different tissues: type A in muscle and erythrocytes, type B in the liver, and type C in the brain. Therefore, drugs that target aldolase will need to be specific for the *T. brucei* aldolase versus the human aldolases. The overall percentage sequence identity between each of the human aldolases and *T. brucei* aldolase is 47%.[7] The crystal structure of *T. brucei* FBA has been determined and shows important similarities with the human enzymes, making the design and synthesis of selective inhibitors challenging.[8] Despite only subtle differences between the FBAs from these two organisms, synthesis of a few selective inhibitors has been reported.[9]

One competitive inhibitor of FBA is hexitol bis-phosphate, which was originally chemically prepared by reduction of fructose bis-phosphate (FBP)[10] as a mixture of diastereoisomers, glucitol bis-phosphate (GBP) and mannitol bis-phosphate (MBP). We previously reported the separate synthesis and biochemical evaluation of these two products.[11] MBP is significantly more active (14-fold) as inhibitor of FBA than the glucitol counterpart. This is in agreement with the structural study of rabbit-muscle FBA crystals soaked with hexitol bis-phosphate solutions, where only the *manno* constituent of the mixture was bound in the active site.[12] MBP interacts strongly with several residues of the mammalian enzyme: the P1-phosphate[13] makes strong hydrogen bonds with Ser 271, Arg 303, Gly 302, and Gly 272 while the P6- phosphate moiety makes hydrogen bonds with Ser 35, Ser 38, and Lys 107 and with the peptide backbone at Glu 34 and Ser 35. The three hydroxyl groups at C-2, C-3, and C-4 also interact with active site residues but, interestingly, not the hydroxyl at C-5, where there seems to be a large empty pocket. The closest residue is situated 5.6 Å from the C-5 hydroxyl. A similar situation is found in *Trypanosoma brucei* FBA, where the putative C-5 binding locus would have a still larger pocket.[14] The absence of active site residues interacting with the C-5 hydroxyl of MBP or in the natural substrate fructose bis-phosphate is consistent with other compounds that are substrates of FBA yet differ in substituents at the C-5 hydroxyl (Figure 1).[15−18] On the basis of this observation, we reasoned that by adding bulky alkyl

Received: May 26, 2011
Accepted: September 2, 2011
Published: September 03, 2011

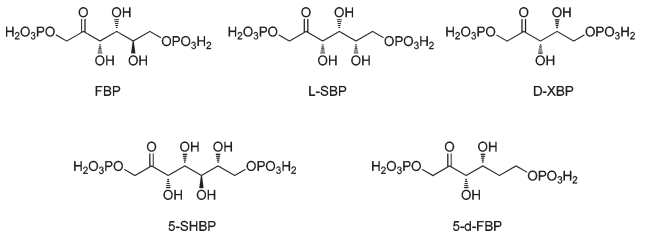


Figure 1. Substrates of FBA with different substituents at C-5 (or a missing hydroxyl) compared to FBP: L-SBP, L-sorbose bis-phosphate; D-XP, D-xylulose bis-phosphate; D-SHBP, D-sedoheptulose bis-phosphate; 5-d-FBP, 5-deoxy-fructose bis-phosphate.[15−18]

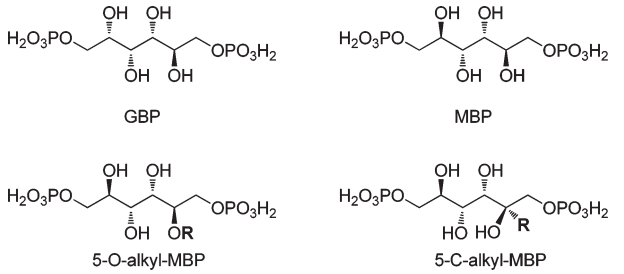
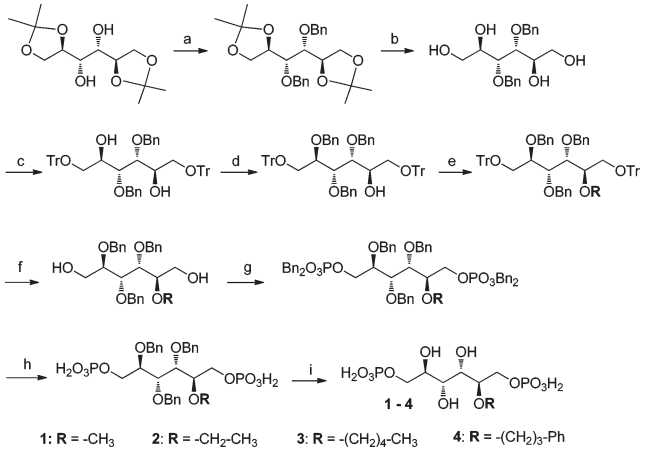


Figure 2. Hexitol bis-phosphate derived inhibitors of FBA.

Scheme 1. Synthesis of 5-*O*-Alkyl Derivatives of Mannitol Bis-phosphate[a]

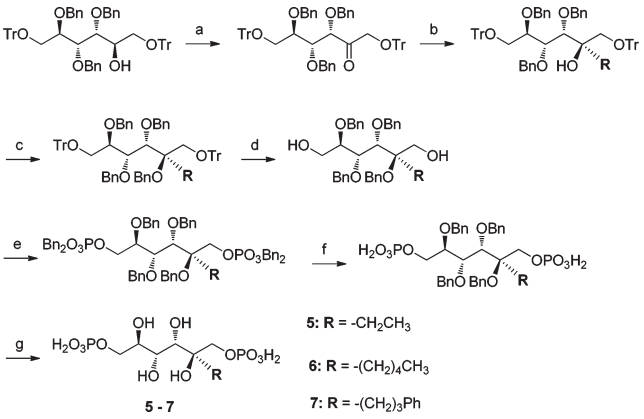


[a] Reagents and conditions: (a) BnBr/NaH/Bu_4NI cat., THF, rt; (b) AcOH/H_2O 7:3, 40 °C, 3 h; (c) TrCl/dimethylaminopyridine, DMF, 25 °C, 18 h; (d) BnBr/NaH 1 equiv/Bu_4NI cat., THF, rt; (e) **R**-Br/NaH, CH_2Cl_2; (f) trifluoroacetic acid/*n*-butanol 1:1, CH_2Cl_2, rt; (g) $^{i}Pr_2NP(OBn)_2$/imidazole/triazole, AcCN, rt, 12 h, then $^{t}BuOOH/H_2O$; (h) H_2/Pd−C, NEt_3, EtOH, then Dowex-50 (H^+); (i) H_2/Pd-C, EtOH.

groups on O-5 or C-5 of MBP it could be possible to prepare selective inhibitors of parasitic FBA unable to enter the active site of the human enzyme (Figure 2). Alternatively, selective irreversible inhibitors bearing an electrophilic group could be conceived.

The first step in this strategy is presented hereby. It consists of validating that derivatives of MBP bearing an alkyl group at O-5 retain their inhibitory capacity.[19] The synthetic strategy of this class of compounds is summarized in Scheme 1.

Scheme 2. Synthesis of 5-*C*-Alkyl Derivatives of Mannitol Bis-phosphate[a]



[a] Reagents and conditions: (a) pyridinium chlorochromate, CH_2Cl_2, rt, 3 h; (b) **R**-MgBr, Et_2O, rt; (c) BnBr/NaH, CH_2Cl_2, rt; (d) trifluoroacetic acid/*n*-butanol 1:1, CH_2Cl_2, rt; (e) $^{i}Pr_2NP(OBn)_2$/imidazole/triazole, AcCN, rt, 12 h, then $^{t}BuOOH/H_2O$; (f) H_2/Pd-C, NEt_3, EtOH, then Dowex-50 (H^+); (g) H_2/Pd-C, EtOH.

Table 1. Enzymatic Inhibition Data Measured in the Presence of Substrate/Inhibitors[21]

	IC_{50} (K_M), μM	
compd	*T. brucei*	rabbit muscle
FBP (substrate)	*10 ± 0.6*	*13 ± 0.8*
MBP	9.4 ± 0.6	8.0 ± 0.5
1	2.0 ± 0.12	7.5 ± 0.5
2	51.0 ± 3	64.0 ± 4
3	117.0 ± 7	157.0 ± 9
4	31.0 ± 2	21.0 ± 1.3
5	130.0 ± 8	244.0 ± 15
6	15.0 ± 1	17.0 ± 1
7	56.0 ± 3.5	137.0 ± 8

As was reported previously, the two-step deprotection to the final product is made necessary by a possible migration of phosphate groups if they were debenzylated after the hydroxyls.[11]

Similarly, the strategy leading to C-5 derivatives starting from the key intermediate of Scheme 1 is given in Scheme 2.[19] The alkylation step involves the reaction of an organometallic reagent on a carbonyl function that could result in a mixture of two diastereoisomers. On the basis of NMR spectra, a single diastereoisomer was obtained in all cases and was assigned the D-manno configuration, in accordance with the Felkin−Ahn rule.

These compounds were tested by standard methods for their inhibitory properties on FBA from rabbit muscle (taken as surrogate model of human FBA, with which it has 98% identity) and on FBA from *Trypanosoma brucei* (His-tagged recombinant enzyme expressed in *E. coli*).[20] The results are reported in Table 1.

Compounds **1**−**7** were shown by double-reciprocal plots to act as competitive inhibitors on *T. brucei* FBA, consistent with results previously obtained on the parent compound MBP. Competitive inhibition for compound **1** is corroborated by the

dx.doi.org/10.1021/ml200129s |*ACS Med. Chem. Lett.* 2011, 2, 804–808

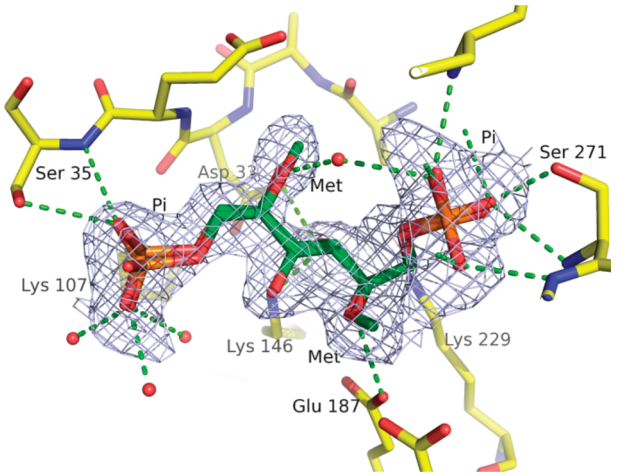


Figure 3. Electron density showing compound **1** trapped in the active site of rabbit muscle aldolase. Compound **1** could be fitted into the electron density using two orientations which are symmetrical with respect to a 2-fold axis of rotation centered on the C3−C4 bond in compound **1**. The electron density encompassing compound **1** was calculated from a simulated annealed $F_o - F_c$ omit map and contoured at 2σ to reveal the electron density of the 5-*O*-methyl, which is at half-occupancy in both orientations. The electron density corresponding to the 5-*O*-methyl group of compound **1** is explicitly labeled (Met) for the two orientations. Phosphate oxyanions are identified as Pi. Hydrogen bonds formed between compound **1** and active site residues are depicted as green dotted lines. Water molecules are shown as red spheres. View is looking into the β-barrel from the carboxyl side of the β-strands.

crystallographic studies. Compared to **1**, inhibitors **2**−**7** show a limited selectivity for Tb-FBA with respect to mammalian-FBA.

A surrogate strategy was undertaken to probe the structural basis for the enhanced discrimination of compound **1** between the two FBA enzymes, and it was the following: The structure of rabbit muscle aldolase in complex with compound **1** was first determined and then modeled by superimposition onto the structure of native *T. brucei* aldolase (PDB entry code 1F2J) to gain structural insight for the affinity differences in compound **1** binding to the two enzymes.

The crystal structure determination of the enzymatic complex formed by soaking rabbit muscle aldolase crystals in the presence of compound **1** is described in the Supporting Information and summarized in Table S1. The crystal structure of the resultant complex formed with compound **1** is shown in Figure 3.

The electron density corresponding to compound **1** binding is continuous and was readily interpreted as compound **1** binding in the active site in two symmetric and fully overlapping orientations, each of equal occupancy that corresponded to full active site occupancy based on *B*-factor comparison with neighboring residues. Compound **1** bound in the active site of rabbit muscle aldolase, and its binding mode was similar with that of MBP, as previously described[12] and shown in Figure 4a.

Compound **1** penetrates slightly deeper into the active site at the 5-*O*-methyl position compared to the binding by MBP; however, it makes no additional contact with active site residues.

Superposition of the rabbit muscle aldolase structure bound with compound **1** onto the crystal structure of *T. brucei* aldolase (rmsd 0.603 Å) revealed that for one binding mode, shown in Figure 4b, the 5-*O*-methyl group of compound **1** would make an additional hydrophobic (van der Waals) contact with the methyl group of active site residue Ala-312.

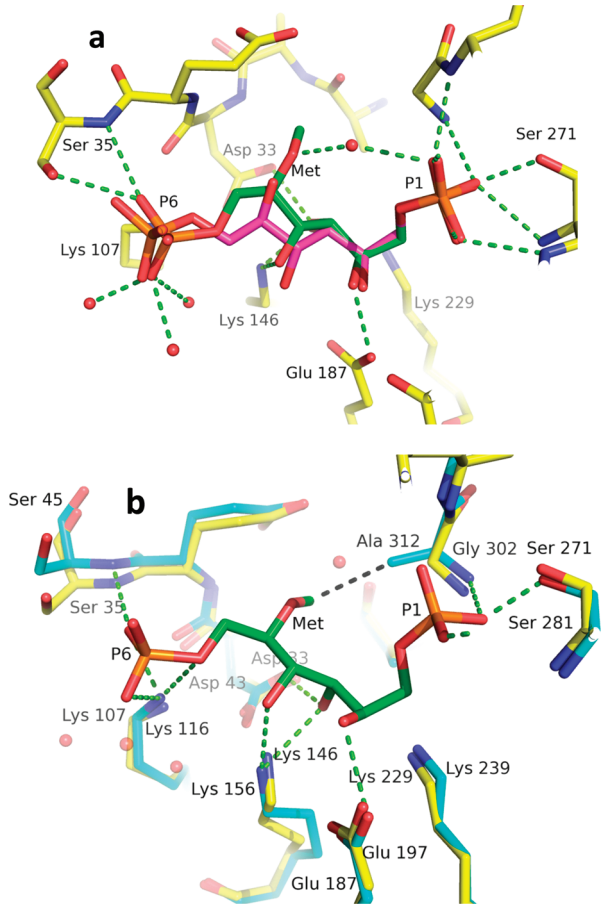


Figure 4. Superimpositions of the crystal structure of rabbit muscle aldolase and *T. brucei* aldolase showing the binding interactions of compound **1**. (a) Comparison of ligand binding in the active site of rabbit muscle aldolase. Superposition of the rabbit muscle aldolase structure bound with compound **1** (green) and that with bound MBP (magenta) (PDB entry code 1ZAJ) yielded an rms value of 0.145 Å consistent with identical subunit conformations. The root mean squared value calculated for identical atoms in both ligands was 0.613 Å. Hydrogen bonding patterns (dashes) made by compound **1** with active site residues were conserved and similar when compared with those in the MBP enzyme adduct. (b) Binding mode of compound **1** in the active site of *T. brucei* aldolase (PDB entry code 1F2J) shown in green. Also shown is subunit A of rabbit muscle aldolase (yellow) that was used to superimpose rabbit muscle aldolase bound with compound **1** onto subunit A of the *T. brucei* aldolase tetramer (rms value of 0.603 Å). The hydrogen bonding patterns (green dashes) made by compound **1** in *T. brucei* aldolase active site were conserved in the two aldolases. The only significant difference was an additional hydrophobic interaction (3.0 Å) made between Ala-312 in *T. brucei* aldolase and the 5-*O*-methyl (Met) in compound **1** that is depicted by a dashed black line. Orientation is similar to Figure 1.

In rabbit muscle aldolase, Gly-302 is the residue equivalent to Ala-312 in *T. brucei* aldolase and does not make close contact with the 5-*O*-methyl of compound **1**. It represents the only amino acid difference between the two active sites and is shown in the alignment of amino acid sequences for rabbit muscle and *T. brucei* aldolases (Table S2 of the Supporting Information). The additional van der Waals interaction made between the 5-*O*-methyl of compound **1** and the Ala-312 methyl group would thus be responsible for the more potent inhibition in *T. brucei* aldolase

by compound **1**, shown in Table 1. The deeper penetration of the aliphatic 5-*O*-methyl group in compound **1** into a region of the active site lined predominantly by aliphatic carbons would not be inconsistent with this interpretation. Furthermore, using the same modeling procedure and superposing the liganded structures of rabbit muscle aldolase bound with FBP and MBP[12] predicts identical interactions by FBP and MBP in *T. brucei* aldolase, and neither ligand makes close contact with Ala-312 (data not shown).

The indistinguishable interactions by FBP and MBP in both aldolases are consistent with their similar K_m and IC_{50} values in both aldolases, shown in Table 1, and corroborate the surrogate modeling strategy. Modeling of compounds **2**, **3**, and **4** using compound **1** as template would result in steric clashes with active site residues in both aldolases, necessitating binding modes that are different from those observed for FBP, MBP, and compound **1** and presumably of lower affinity, as shown in Table 1. Similarly, modeling of the binding modes for compounds **5**, **6**, and **7** based on that of compound **1** could not be accommodated due to steric clashes in the active site of both aldolases and consistent with active site interactions resulting in poorer affinity by these compounds, as seen in Table 1.

The present selectivity, not exceeding a factor of 4, is obviously too small for therapeutic utilization. One positive result, however, is that MBP derivatives bearing bulky groups at O-5 (C-5) retain appreciable inhibitory properties. Modeling of the 5-*O*-CH_2F group in compound **1** suggests binding by such a compound upon active site adjustment; however, the gain in interaction energies rarely exceeds 1 kcal/mol.[22] In this regard, derivatives bearing short reactive functions at C-5 could be introduced to act as irreversible selective inactivators. The proposed studies would take advantage of docking calculations of the compounds bound in the active site of the two enzymes and followed up by structure determination of appropriate enzyme−inhibitor complexes. Additional studies to be performed would include the use of mutational analyses in concert with structural and/or kinetic data to corroborate the role of active site residues responsible for selectivity, as described previously.[23]

■ ASSOCIATED CONTENT

Supporting Information. Detailed syntheses and characterizations of compounds **1**−**7**, and crystallographic experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

C.-G.M-B.: Chemical syntheses, kinetic measurements; G.A-C.: Crystallographic studies; V.H.: preparation of recombinant T.b. FBA; H.T.: kinetic measurements, preparation of enzymes; J.S.: Crystallographic studies, preparation of manuscript; M.T.: kinetic measurements, preparation of manuscript, and supervision of C.-G.M.-B. Ph.D.

Funding Sources

This work was supported by a scholarship to C.-G.M.-B. from Government of the République du Congo. J.S. was supported by the Natural Science and Engineering Research Council (Canada) and Canadian Institutes for Health Research.

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