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Designing Inhibitors Against Fructose 1,6-bisphosphatase: Exploring Natural Products for Novel Inhibitor Scaffolds

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Abstract

Natural products often contain unusual scaffold structures that may be elaborated by combinatorial methods to develop new drug-like molecules. Visual inspection of more than 128 natural products with some type of anti-diabetic activity suggested that a subset might provide novel scaffolds for designing potent inhibitors against fructose 1,6-bisphosphatase (FBPase), an enzyme critical in the control of gluconeogenesis. Using in silico docking methodology, these were evaluated to determine those that exhibited affinity for the AMP binding site. Achyrofuran from the South American plant *Achyrocline satureioides*, was selected for further investigation. Using the achyrofuran scaffold, inhibitors against FBPase were developed. Compounds **15** and **16** inhibited human liver and pig kidney FBPases at IC₅₀ values comparable to that of AMP, the natural allosteric inhibitor.

Keywords

Fructose 1,6-bisphosphatase; Type 2 diabetes; Anti-diabetic; Drug design; Natural products

1. Introduction

Significant effort has been brought to bear towards the development of new therapeutics for the treatment of type-2 diabetes, specifically through the use of small molecule compounds binding to an enzyme target. A number of these have targeted fructose-1,6-bisphosphatase (FBPase, EC 3.1.3.11), an enzyme critically involved in the control of gluconeogenesis [1-4]. In type-2 diabetes, the enzymatic control of gluconeogenesis is compromised, thus allowing the production of excessive amounts of glucose resulting in elevated blood glucose levels characteristic of the disease. In normal individuals, when blood glucose levels are high there is a corresponding increase in the concentration of fructose 2, 6-bisphosphate, a potent competitive inhibitor of FBPase [5]. The increase in cellular concentrations of fructose 2, 6-bisphosphate is hormonally regulated by elevated insulin concentration. However, the biology of type-2 diabetes patients is insulin resistance.

FBPase is an allosteric enzyme composed of four identical chains (M_r 36,700) organized as a dimer of dimers. Regulation of enzymatic activity involves changes in quaternary structure

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between the active (R) and inactive (T) conformational states [6,7]. Metal cations, fructose 1, 6-bisphosphate, and fructose 6-phosphate stabilize the R state, while AMP binds to the allosteric site and inhibits the enzyme by shifting the enzyme from the R to the T conformation. Fructose 2, 6-bisphosphate, a potent competitive inhibitor of FBPase, synergistically increases the binding affinity of AMP [4]. As a drug target leading to inhibition of FBPase, there are three possible sites for small molecule binding: (i) the active site, which binds the substrate fructose 1, 6-bisphosphate (FBP) and the natural inhibitor fructose 2, 6-bisphosphate, (ii) the allosteric site, which binds AMP, and (iii) a 'novel' allosteric site [8] located at the interface between the four monomers.

Considerable research has been focused on designing small-molecule inhibitors of FBPase, since patients with hereditary fructose 1,6-bisphosphatase deficiency seem to develop normally after early childhood [9]. Wright et al. [10] reported a series of anilinoquinazolines as allosteric inhibitors of FBPase, identified by screening a library of compounds. However, they found that these anilinoquinazolines bound to a 'novel allosteric site'. Another class of inhibitors identified by a high-throughput screen (HTS), which also bind to the 'novel allosteric site', is a group of benzoxazole-2-benzene-sulfonamides [11]. Other small-molecule inhibitors, which bind to the allosteric site of FBPase and are competitive with the binding of AMP, include MB05032 [12] and 3-(2-carboxy-ethyl)-4,6-dichloro-1H-indole-2-carboxylic acid [13].

The popularity of HTS as an approach to drug discovery is no doubt due to the ability to test a large number of potential effectors of biological activity against a biological event or an enzyme target such as FBPase. As an alternative to HTS to identify novel FBPase inhibitors and/or new scaffolds, we decided to embark on a less traveled but nonetheless relevant path, by focusing our attention on natural products to provide us with novel scaffolds for our inhibitor design. Given the plethora of natural products whose structures are known, finding the class of natural products with the right scaffold is not trivial. Hence, we decided to concentrate our focus by examining natural products that are reported to possess anti-diabetic properties. The use of natural products to treat diabetes is not a new concept. Long before the advent of insulin, symptoms manifested from what we now know as diabetes were treated with plant medicines. In fact, natural products from more than 1,200 species have been reported as being used for the treatment of diabetes [14]. And hence, we speculate that there is a high probability that some of these natural products may function via the inhibition of FBPase, and if so, would give us leads which we can then develop into novel inhibitors against the enzyme.

Zareba et al. [15] summarized 128 natural products of known structure with anti-diabetic activity. Here, we evaluated the interactions of these natural products to the allosteric site of human FBPase using in silico docking methods. We selected achyrofurane (2, Figure 1) for further studies because of a number of reasons: (i) the in silico docking indicated that achyrofurane bound to the AMP site of FBPase with high affinity, (ii) Achyrocline satureioides, in which achyrofurane is the active agent responsible for its antihyperglycemic property, is used as a treatment for lowering blood sugar in humans [15] (iii) achyrofurane has proven potency as an anti-diabetic agent in mouse models [16] and (iv) achyrofurane has 'drug-like' properties according to the Lipinski's rule of 5 [17].

2. Results and Discussion

2.1. In Silico Docking

Zareba et al. [15] divided 128 natural products, with known structures and anti-diabetic activity, into 16 structural groups and conveniently provided 16 diverse natural product scaffolds as leads. We began by evaluating the interactions of these natural products to the

allosteric site of human FBPase by in silico docking methods, using the GLIDE program [18,19]. GLIDE was chosen as the main docking software because it proved to be more accurate than SURFLEX and twice as accurate as GOLD when the docking accuracy for GLIDE was assessed by redocking ligands from 282 cocrystallized PDB complexes [18].

We validated GLIDE for use with FBPase by three separate methods. First, we docked AMP into the structure of the human enzyme with AMP bound (PDB entry 1FTA) [20]. The receptor was setup by removing the AMP molecule from its site, and AMP was setup initially so that it was in a different spatial position, distant from the actual binding site. AMP was then docked into the allosteric site using the high-precision mode (XP) of GLIDE. GLIDE performed well in matching the docked conformation to the one observed in the crystal structure. The root mean square (RMS) deviation between the position of AMP in the crystal structure and that obtained by GLIDE docking was 0.38 Å.

The second method used to validate the use of GLIDE with FBPase was to dock compound **4** into the pig kidney FBPase structure (PDB entry 1LEV). This structure has **4** bound in the allosteric site of the enzyme [13]. Compound **4** was one of a set of indole carboxylic acid compounds identified via a HTS against human FBPase [13]. Wright et al. [13] reasoned that the reported structure was a good model for the complex with the human enzyme, since the degree of amino acid sequence conservation within mammalian FBPases is very high (>95% identical or similar).

The RMS deviation between the position of **4** in the X-ray structure of the pig kidney FBPase (PDB entry 1LEV) and that determined by docking using the high-precision mode (XP) of GLIDE to that structure was 1.07 Å. This value of the RMS deviation indicated that the position obtained by docking was very similar to that observed in the X-ray structure. When **4** was docked into the allosteric site of human FBPase (PDB entry 1FTA) the conformation and interactions observed in the human enzyme were again similar to those observed in the structure of the pig kidney enzyme and overlapped the position of AMP (see Figure 2).

The third method used to validate GLIDE was to determine the relative binding affinities of a series of known FBPase that bind to the allosteric site (Figure 1). The calculations were performed using the high-precision mode (XP) of GLIDE [19]. The resulting trend in the GLIDE XP docking score, as defined by Halgren et al. [19], correlated (correlation coefficient $r = 0.9$) well to the log of the published IC_{50} values (see Figure 3).

Of the identified chemical compounds, known to be active anti-diabetic constituents from plants [15], seventy-one docked into the allosteric site of the human FBPase structure, and the best-docked conformation of each was evaluated. Of the compounds docked, compound **2** was of particular interest, because when diabetic db/db mice were dosed orally with pure **2** at 20 mg/kg once per day, **2** was able to lower the serum glucose levels of these mice below those in the positive control group treated with Metformin, dosed at 250 mg/kg once per day [16]. Compound **2** was originally isolated from *Achyrocline satureioides*, using a bioassay-guided fractionation using a diabetic db/db mouse model [16]. *A. satureioides*, a medium-sized aromatic herb that grows throughout Brazil, Uruguay and Argentina, has been used in herbal medicine throughout this region to treat a variety of ailments including type-2 diabetes. In addition, **2** also has 'drug-like' properties according to the Lipinski's rule of 5 [17] and bound, in silico, to the AMP site of human FBPase with an affinity that is comparable to that of AMP.

Our docking results indicate that **2** makes a total of 14 contacts within the AMP binding site of FBPase, five hydrogen-bonding interactions and nine hydrophobic interactions. Compound **2** forms hydrogen bonding interactions with Thr27, Gly28, Glu29, Lys112 and

Tyr113 and hydrophobic interactions with Gly21, Arg22, Gly26, Thr31, Arg140, Val 160, Met177, Asp178 and Cys179 (Figure 4). In addition, **2** fills the space within the allosteric binding pocket better than AMP (Figure 5).

In order to support the hypothesis that a structural scaffold based on **2** can be an inhibitor of FB Pase, we performed a substructure search to find a purchasable compound, with a structural scaffold that is as similar as possible to **2**, to test in vitro. From this search (+)-usnic acid (**3**), a naturally occurring dibenzofuran derivative found in several epiphytic lichen species was identified. The high-precision mode (XP) of GLIDE [19] was used to dock **3** into the allosteric site of FB Pase. The results from GLIDE indicated that **3** had reasonable affinity for the allosteric site, although significantly less than **2**. Activity measurements showed that **3** was able to inhibit FB Pase in vitro. As seen in Table 1, compound **3** inhibited both human liver and pig kidney FB Pases with IC₅₀ values of 0.37 and 0.93 mM, respectively.

2.2. Chemistry

We began by designing analogues bearing most of the functional entities found on achyrofuran. The rationale being that if these analogues inhibited both human liver and pig kidney FB Pases, then it would provide strong evidence that the achyrofuran scaffold can be used to generate novel and potent FB Pase inhibitors. Based upon the modeling of about twenty analogues, we selected **15** and **16** as our first synthetic targets because they (i) docked with similar affinity to FB Pase as **2**, and (ii) could be readily synthesized using modifications of published procedures. Since stereochemistry at the three stereogenic centers of achyrofuran has not yet been determined, **15** was designed to possess no chiral centers, while **16** was synthesized as a mixture of stereoisomers. We removed the methypentenol and methylpentene groups, so that we could design analogs with improved hydrophilicity.

The synthesis of compounds **15** and **16** is illustrated in Scheme 1. The synthesis of **12** was accomplished by the single iodination of commercially available 1,3,5-trimethoxybenzene (**11**) using solid iodine in the presence of iodic acid, as previously described with modifications [21]. Since 1,3,5-trimethoxybenzene is a symmetrical molecule, the reaction was allowed to proceed freely without the need to control the position for substitution. Next, the biphenyl intermediate **13** was synthesized by an Ullmann coupling. Conventionally, the Ullmann reaction is carried out in the presence of copper bronze [21,22]. However, we found that by substituting solid copper for the copper bronze the yields were substantially improved. Cyclization and the concomitant deprotection of **13** in a 1:1 mixture of HBr and acetic acid [23,24] conveniently yielded **14** as the key intermediate. Next, we were faced with the challenge of functionalizing **14** with the appropriate acyl moiety. Attempts to introduce the isovaleryl group on the ring with isovaleryl chloride and tin (IV) chloride [25], as well as with isovaleric acid and boron trifluoride [22], proved unsuccessful. We reasoned that the isovaleryl portions could be strategically placed by means of a Friedel Crafts reaction to ultimately yield **15**. ¹H chemical shifts of the two dibenzofuran protons at δ 6.77 and 7.27, respectively, proved that the Friedel Crafts acylation with AlCl₃ had successfully placed the isovaleryl functionalities on opposing sides of the ring. Having successfully synthesized **15**, the same strategy was applied in synthesizing **16** by simply substituting isovaleryl chloride with 2-methylbutyryl chloride.

2.3. In vitro assays of **15** and **16** against pig kidney and human FB Pase

Compounds **15** and **16** were tested to determine if they could inhibit either pig kidney and human liver FB Pase using a coupled-enzyme assay [26] using the natural allosteric inhibitor AMP as a control. Compound **15** was found to inhibit pig kidney FB Pase with an IC₅₀ of 1.5

μM as compared to $1.3 \mu\text{M}$ for AMP, while the same compound inhibited human liver FBPase with an IC_{50} of $8.1 \mu\text{M}$ as compared to $9.7 \mu\text{M}$ for AMP. Compound **16**, containing the 2-methylbutyryl functionality, inhibited the pig and human FBPases with IC_{50} values of 5.0 and $6.0 \mu\text{M}$, respectively. (+)-Usnic acid inhibited the enzyme with IC_{50} values of 930 and $371 \mu\text{M}$ while the dibenzofuran scaffold **14** did not inhibit either enzyme. The ability of (+)-usnic acid to inhibit the enzymes, might be due to the presence of the carbonyl moieties at C1 and C1'. The carbonyl functionalities are an integral part of achyrofurans and are also present in **15** and **16** while being absent in **14**.

In order to confirm that the FBPase inhibition by the achyrofurans was due to binding at the allosteric site and not the active site, a competition experiment was performed using the AMP analogue, 2',3'-O-(2,4,6-trinitrophenyl)adenosine 5-monophosphate (TNP-AMP). This analogue has been shown to bind at the allosteric site of FBPase and exhibits fluorescence only when bound to the enzyme [27]. In separate experiments, TNP-AMP was added to pig kidney and human liver FBPase at a concentration equal to 0.5 times their respective IC_{50} values for AMP. Subsequently, increasing concentrations of **15** or **16** were added to the above solution, which resulted in a substantial diminution of the TNP-AMP fluorescence, indicating that both **15** and **16** were competing with TNP-AMP at the allosteric site. Binding constants for **15** and **16** could not be determined by this method due to the relatively low solubility of these compounds.

3. Conclusion

Natural products and their derivatives have historically been invaluable as a source of therapeutic agents. Of the 877 small-molecule New Chemical Entities (NCEs) introduced between 1981 and 2002, approximately 49% were either natural products, semi-synthetic natural-product analogs or synthetic compounds based on natural-product pharmacophores [28]. This, together with the notion that natural-product structures have long been recognized to possess characteristics of high chemical diversity, biochemical specificity and molecular diversity within the boundaries of reasonable drug-like properties, make them attractive targets as lead structures for drug discovery [29]. Here we employed *in silico* docking methods to determine which of the known anti-diabetic natural products can potentially inhibit FBPase so as to increase the probability of finding a potential lead. In this present work, we chose to focus on achyrofurans based on the reasons illustrated in the paper. As such, achyrofuran analogs **15** and **16** were synthesized and these were found to inhibit both pig kidney and human liver FBPase with comparable IC_{50} values to those of the natural allosteric inhibitor AMP. Furthermore, the data suggest that **15** and **16** can compete with AMP for its binding site, indicating that **15** and **16** are new allosteric inhibitors of these enzymes. In addition, the fact that **15** and **16** follow Lipinski's rule of five [17] suggest that the achyrofuran scaffold has the potential to be developed into a new class of anti-diabetic drugs. Although we only concentrated on achyrofurans in this work, many of the other anti-diabetic compounds docked as potential allosteric FBPase inhibitors and we are currently examining others which we think can be pursued as new drug scaffolds in the future based upon our findings.

4. Experimental

4.1. Molecular modeling

Docking of AMP and inhibitors to human liver and pig kidney FBPase was performed using Glide (Schrödinger, Inc.) [18,19] in the high-precision (XP) mode. The necessary grids were prepared based on structures in the Protein Data Bank (PDB), namely 1FTA [20] and 1EYK [30] for the human and pig enzymes, respectively. The coordinates of AMP in the respective structures were used as the center of the grid box. 3D structures of AMP and inhibitors were

generated using Maestro (Schrödinger, Inc.), and then processed through LigPrep (Schrödinger, Inc.), to produce a number of versions of the inhibitor structures with various ionization states, tautomers and stereochemistries as necessary for each case.

4.2. Isolation and purification of human liver FBPase

An expression plasmid (pEK694) for human liver FBPase was constructed by inserting the human liver FBPase gene from plasmid EX-C0133-B31 (GeneCopoeia) into the vector pET23a (Novagen, Inc.). The FBPase gene from EX-C0133-B31 had a C-terminal 6xHis tag for use in purification.

For expression of the human liver FBPase, chemically competent BL21(DE3) Rosetta cells (Novagen, Inc.) were transformed with pEK694. A 5 mL overnight culture grown in LB media [31] with 150 µg/mL of ampicillin was back-diluted 1000-fold into 2 L of 2YT [31] media with 0.4% glycerol and 150 µg/mL ampicillin. Bacterial growth at 37°C was monitored using the A₅₆₀ and the doubling-time was calculated. When the growth reached an A₅₆₀ of 0.4, 0.1 mM IPTG was added to induce the cell, following which the cells were allowed to grow for three additional doubling times. The cells were then harvested by centrifugation (4400 x g), resuspended in 5 mM K₂HPO₄, pH 7.4, 2.5 µg/mL leupeptin, 0.1 mg/mL lysozyme and 50 mM imidazole and lysed by sonication. The lysate was clarified by centrifugation at 31,000 x g for 15 min. The protein was purified through a Chelating Sepharose Fast Flow column (GE Healthcare), which had been charged with 0.3 M NiSO₄. Proteins that did not bind to the column were eluted with 10 column volumes of 50 mM imidazole, pH 7.5. The human liver FBPase was then eluted with buffer containing 50 mM sodium citrate and 50 mM NaCl, pH 4.0. The standard elution procedure using high concentrations of imidazole could not be employed, since the enzyme precipitated under these conditions. The protein-containing fractions were pooled and dialyzed against 50 mM Tris-acetate buffer, 150 mM NaCl, pH 7.5 at 4 °C overnight. Protein purity was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis [32]. Protein concentration was determined using the BioRad version of the Bradford dye-binding assay [33] with bovine serum albumin as the standard.

4.3. Isolation and purification of pig kidney FBPase

In order to purify the pig kidney FBPase more efficiently, by metal chelating chromatography, six His residues were added to the C-terminal of the enzyme. This was accomplished by mutating the last two codons of the pig kidney FBPase sequence in plasmid pEK284 [34] to a XhoI site, by site-specific mutagenesis. The new plasmid was then digested with XhoI and the resulting fragments separated on an agarose gel. The larger fragment (4.6 Kb) was purified from the agarose gel and then religated by adding T4 DNA ligase at 12° C for 12 hrs. The new plasmid pEK324 had the pig kidney coding sequence with the last two codons replaced with the codons for Leu-Glu-His-His-His-His-His. Competent E. coli BL21(DE3) cells (Novagen, Inc.) were transformed with pEK324 and grown in YT [31] media at 37 °C, containing 150 µg/mL ampicillin. Bacterial growth at 37°C was monitored at 560 nm and the doubling-time was calculated. When the growth reached an OD at 560 nm of 0.7, cells were induced with 0.4 mM IPTG and allowed to grow for an additional 18 h. The cells were harvested and purified according to the method described above for the human liver FBPase.

4.4. Measurement of FBPase activity

FBPase activity was measured spectrophotometrically by employing the coupling enzymes phosphoglucose isomerase and glucose-6-phosphate dehydrogenase [26]. The reduction of NADP⁺ to NADPH was monitored directly at 340 nm. Specifically, buffer (0.2 M Tris, 4 mM MgCl₂, 4 mM (NH₄)₂SO₄, 0.1 EDTA, pH 7.5), 0.2 mM of NADP⁺, 1.4 units of

phosphoglucose isomerase and 0.5 units of glucose-6-phosphate dehydrogenase, 0 – 300 μM of inhibitor and 9 ng of FBPase, were mixed in a cuvette and equilibrated at 30° C. 70 μM of FBP was then added to initiate the reaction. A_{340} data were collected as a function of time, using a JASCO V-630 spectrophotometer. After a lag phase due to coupling, a straight line was fitted to the progress curve and the slope value was recorded as ΔA_{340} per min. The amount of NADPH produced per minute was calculated using the 340 nm millimolar extinction coefficient of 6.22. At each inhibitor concentration, reactions were performed in duplicate. Inhibition curves were obtained for each compound by plotting the relative activity versus inhibitor concentration.

4.5. Fluorescence measurements

Fluorescence data were collected using a JASCO FP-6300 spectrofluorometer. Excitation and emission wavelengths of 410 and 535 nm respectively were used for TNP-AMP (Molecular Probes). 0.15 mg of the respective FBPases, 0.08 mM MgCl_2 and 20 μM fructose 1,6-bisphosphate in 50 mM Tris-acetate buffer, pH 7.5, was added to a 2 mL cuvette and stirred. 3.5 μM of TNP-AMP was added to the cuvette and the fluorescence emission was measured. Microliter volumes of **15** were added sequentially with the fluorescence emission recorded after each addition. The final concentration of **15** in the enzyme solution was 6 μM . The competition experiment for **16** was performed in the same manner as described for **15**.

4.6. Materials and methods

All materials were reagent grade and used without further purification. Thin-layer chromatography (TLC) was performed on silica gel 60 aluminum backed plates (250 microns) from Sorbent Technologies and visualized by A_{254} irradiation. Flash chromatography was conducted with silica gel 60 (230 mesh) from AK Scientific Inc. ^1H NMR spectra were recorded on a Varian 400 spectrometer. Proton chemical shifts are reported in ppm (δ) relative to internal tetramethylsilane (TMS, δ 0.0) or with the solvent reference relative to TMS employed as the internal standard (CDCl_3 , δ 7.24 ppm; $\text{DMSO}-d_6$, δ 2.50). Standard abbreviations indicating multiplicity were used as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet and br = broad. Mass spectra were obtained at the Mass Spectrometry Facilities at Boston College. HPLC separations were performed on a Biologic Duo Flow chromatography system (BioRad).

4.6.1. 2-Iodo-1,3,5-trimethoxybenzene (12)—1,3,5-Trimethoxybenzene (14.5 g, 0.086 mol) was dissolved in 10 mL of ethanol at rt before warming to 50° C in a waterbath after which, an aqueous solution of iodic acid (7.6 g, 0.043 mol) was added. Solid iodine (21.88 g, 0.086 mol) was dissolved in 20 mL of ethanol and the resultant solution was added portion-wise over 60 min to the reaction mixture. Stirring was continued at 50° C until the color of I₂ faded and a white precipitate appeared. The reaction mixture was stirred at rt for 18 h. The precipitate that formed was isolated via vacuum filtration, washed with ethanol and crystallized from methanol to yield **12** (21.7 g, 86%) as white crystals. m.p. 120 – 122° C (Lit. 119 – 121° C [21]); ^1H NMR (400 MHz, $\text{DMSO}-d_6$): δ 3.80 (s, 9H, overlapped, 2 x ICCOCH_3 and CHCOCH_3), 6.28 (s, 2H, CCHC); ^{13}C NMR ($\text{DMSO}-d_6$, 100 MHz) 54.9, 55.5, 91.1, 116.5, 158.6, 160.0; ESI-HRMS $[\text{M} + \text{H}]^+$: calc. for $\text{C}_9\text{H}_{12}\text{IO}_3$: 294.9831, found: 294.9842.

4.6.2. 2,2',4,4',6,6'-Hexamethoxybiphenyl (13)—Compound **12** (7.0 g, 0.024 mol) and copper powder (2.0 g, 0.031 mol) were mixed thoroughly and transferred to a heavy wall, pressure vessel. The vessel was placed into a sand-bath at the initial temperature of 120° C. Once the vessel was secured, the temperature of the bath was raised to 235° C in 15 min and kept at 235° C for 1 h. The vessel was allowed to cool and the contents removed

and powdered. The product was extracted with methanol for 18 h in a soxhlet. The resultant solution was concentrated in vacuo. Purification of the crude reaction mixture via column chromatography (1:5 ethyl acetate/hexane) yielded **13** (3.1 g, 78%) as a white solid: m.p: 155 – 157° C (Lit. 156° C [21]); ¹H NMR (400 MHz, DMSO-*d*₆): δ 3.58 (s, 12H, 4 x CCOCH₃), 3.78 (s, 6H, 2 x CHCOCH₃), 6.21 (s, 4H, 4 x CHCOCH₃); ¹³C NMR (DMSO-*d*₆, 100 MHz) 55.1, 55.4, 90.5, 92.8, 158.7, 161.1; ESI-HRMS [M + H]⁺: calc. for C₁₈H₂₃O₆: 335.1495, found: 335.1502.

4.6.3. 1,3,7,9-Tetrahydroxydibenzofuran (14)—Compound **13** (3 g, 8.97 mmol) was refluxed for 18 h in a 20 mL solution consisting of a 1:1 mixture of HBr and acetic acid. The reaction mixture was neutralized by adding saturated aqueous NaHCO₃. The subsequent mixture was then extracted with ethyl acetate and the organic layer was further washed with brine, water and dried over Na₂SO₄. After removing the solvent in vacuo, the brown, viscous residue was purified by column chromatography (5-20 % ethyl acetate/hexane) to yield **14** (1.6 g, 77%) as a yellow solid: m.p: 94 – 96° C; ¹H NMR (400 MHz, DMSO-*d*₆): δ 6.23 (s, 2H, OHCCCHCOH), 6.45 (s, 2H, OCCHCOH), 9.59 (s, 2H, 2 x OHCCCHCOH), 10.18 (s, 2H, 2 x OCCHCOH); ¹³C NMR (DMSO-*d*₆, 100 MHz) 90.5, 97.3, 115.1, 149.1, 156.6, 157.2; ESI-HRMS [M + H]⁺: calc. for C₁₂H₉O₅: 233.0450, found: 233.0459.

4.6.4. 1,1'-(1,3,7,9-tetrahydroxydibenzofuran-2,6-diyl)bis(3-methylbutan-1-one) (15)—Compound **14** (100 mg, 0.431 mmol) and aluminum trichloride (287 mg, 2.15 mmol) were suspended in 5 mL of carbon disulfide at rt. Chloroform (6 mL) was added to the suspension and the reaction mixture was warmed to 50° C in a water-bath. Isovaleryl chloride (259 mg, 2.15 mmol) was added dropwise and the reaction was stirred for 2 h at 50° C. After removing solvent in vacuo, and the crude reaction mixture was purified by column chromatography (1-5% ethyl acetate/hexane) to yield **15** (30 mg, 17%) as a white solid: ¹H NMR (400 MHz, CDCl₃): δ 1.04 -1.07 (m, 12H, 4 x CH₂CHCH₃), 2.23 – 2.26 (m, 2H, CH₂CHCH₃), 2.44 (d, *J* = 7.2 Hz, 2H, OHCCCCCH₂), 2.54 (d, *J* = 6.8 Hz, 2H, OCCCCCH₂CHCH₃), 6.77 (s, 1H, CCHC), 7.27 (s, 1H, OCCHC); ¹³C NMR (CDCl₃, 100 MHz) 23.0, 24.1, 26.4, 51.1, 54.2, 87.7, 101.1, 108.9, 113.2, 117.2, 117.3, 152.3, 155.8, 156.5, 157.8, 160.4, 163.7, 200.1, 205.8; ESI-HRMS [M + H]⁺: calc. for C₂₂H₂₅O₇: 401.1600, found: 401.1614.

4.6.5 1,1'-(1,3,7,9-tetrahydroxydibenzofuran-2,6-diyl)bis(2-methylbutan-1-one) (16)—Compound **16** was synthesized according to the same procedure as described for **15**, by substituting isovaleryl chloride with 2-methylbutyryl chloride (3.36 mmol, 429.0 μL) to yield the desired product (40 mg, 15%) as white solid: ¹H NMR (400 MHz, CDCl₃): δ 1.00 – 1.06 (m, 6H, CCCHCH₃ and CCCHCH₂CH₃ overlapped), 1.29 (d, *J* = 6.8 Hz, 3H, OCCCCCHCH₃), 1.33 (dd, *J* = 7.0, 4.2 Hz, 3H, OCCCCCHCH₂CH₃), 1.60–1.66 (m, 2H, CCCHCH₂CH₃), 1.80–1.87 (m, 2H, OCCCCCHCH₂CH₃), 2.63 – 2.74 (m, 2H, CCCHCH₃ and OCCCCCHCH₂CH₃ overlapped) 6.74 (s, 1H, CCHC), 7.26 (s, 1H, OCCHC). ¹³C NMR (CDCl₃, 100 MHz) 12.2, 15.3, 15.7, 25.0, 25.6, 43.8, 47.9, 95.5, 100.1, 105.2, 113.3, 114.5, 115.1, 160.2, 152.6, 158.5, 161.4, 164.7, 202.3, 212.4. ESI-HRMS [M+H]⁺: calc. for C₂₂H₂₅O₇: 401.1600, found: 401.1611.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Structures of FBPase inhibitors. AMP (1), Achyrofuran (2), (+)-Usnic acid (3), Inhibitors (4) [13], (5), (6) [35], (7) [36], (8) [37], (9) [11] and (10) [12] are known inhibitors of FBPase that were docked against the enzyme using GLIDE.

Fig. 2. Comparison of the docked position of **4** with AMP in the crystal structure. Compound **4** (sticks) was docked into the allosteric site of human FBPase (PDB code 1FTA) and the best-docked conformation was overlapped with AMP (CPK). The docked conformation of **4** overlaps the position occupied by AMP [20]. This figure was created using PyMol [38].

Fig. 3.

Correlation between experimentally observed IC_{50} values for FBPase inhibitors and the GLIDE XP Score. AMP (1) and known inhibitors of FBPase were docked into the allosteric site of human FBPase (PDB entry 1FTA) and the GLIDE high-precision XP score calculated. The numbers associated with the data points in this figure correspond to the compounds in Figure 1. A more negative docking score represents tighter binding, and therefore should predict better inhibition.

Fig. 4. Interactions of achyrofuran with FBPase. (Top) **2** bound in the allosteric site of FBPase (PDB code 1FTA). The protein-ligand interactions were determined using Ligplot [39]. (Bottom) Schematic representation of **2** bound in the allosteric site of FBPase in the same orientation as shown in the top part of the figure.

Fig. 5. Comparison of AMP and achyrofuran bound to FBPase. (a) AMP bound in the allosteric site of human FBPase as determined by X-ray crystallography (PDB entry 1FTA, chain A). (b) Achyrofuran (**2**) docked in the allosteric site of human FBPase using the high-precision XP mode of Glide (Schrödinger). This figure was produced using PyMol [38].

Scheme 1.

The synthesis of inhibitors **15** and **16**. (a) I₂, HIO₃, EtOH, reflux, 18 h (b) Cu(s), 235 °C, 1 h (c) HBr, AcOH, reflux, 18 h (d) acid chloride, AlCl₃, CS₂, CHCl₃, 50 °C, 1 h.

Table 1IC₅₀ Values of Human Liver and Pig Kidney FBPase Intermediates and Inhibitors.^[a]

Compound	Pig kidney FBPase IC ₅₀ (μM)	Human liver FBPase IC ₅₀ (μM)
AMP	1.3 ± 0.1	9.8 ± 0.1
Usnic Acid	930 ± 11	371 ± 13
15	1.5 ± 0.1	8.1 ± 0.8
16	5.0 ± 0.3	6.0 ± 0.3
14	ND ^[b]	ND ^[b]

^[a] Activity measurements were determined at 30 °C in 0.2 M Tris-acetate, 4 mM MgCl₂, 4 mM (NH₄)₂SO₄, 0.1 EDTA buffer pH 7.5.^[b] No inhibition observed.