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Rational Design, Synthesis, and Potency of N-Substituted Indoles, Pyrroles, and Triarylpyrazoles as Potential Fructose 1,6-Bisphosphatase Inhibitors

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By using computer modeling and lead structures from our earlier SAR results, a broad variety of pyrrole-, indole-, and pyrazole-based compounds were evaluated as potential fructose 1,6-bisphosphatase (FBPase) inhibitors. The docking studies yielded promising structures, and several were selected for synthesis and FBPase inhibition assays: 1-[4-(trifluoromethyl)-benzoyl]-1*H*-indole-5-carboxamide, 1-(α-naphthalen-1-ylsulfonyl)-7-nitro-1*H*-indole, 5-(4-carboxyphenyl)-3-phenyl-1-[3-(trifluoromethyl)phenyl]-1*H*-pyrazole, 1-(4-carboxyphenylsulfonyl)-1*H*-pyrrole, and 1-(4-carbomethoxyphenylsulfonyl)-1*H*-pyrrole were synthesized and tested for inhibition of FBPase. The IC_{50} values were determined to be 0.991 and 1.34 μM, and 575, 135, and 32 nM, respectively. The tested compounds were significantly more potent than the natural inhibitor AMP (4.0 μM) by an order of magnitude; indeed, the best inhibitor showed an IC_{50} value toward FBPase more than two orders of magnitude better than that of AMP. This level of activity is virtually the same as that of the best currently known FBPase inhibitors. This work shows that such indole derivatives are promising candidates for drug development in the treatment of type II diabetes.

Introduction

Owing to the increasing rate of obesity worldwide, the so-called type II or non-insulin-dependent diabetes is becoming one of the most frequently diagnosed diseases. As the number of individuals with impaired glucose tolerance in the pre-diabetic state is steadily rising, the inhibition of fructose 1,6-bisphosphatase (FBPase), as one of the principal treatment options, continues to attract significant attention.[1] In recent years many new potential therapeutics have been screened and identified to inhibit gluconeogenesis by blocking FBPase activity. Some of these compounds have excellent IC_{50} values, such as MB05032 ($IC_{50}=16$ nM),[2] benzimidazole phosphonic acid ($IC_{50}=90$ nM),[3] and 10A ($IC_{50}=16$ nM).[4] Most drug candidates that reached early clinical trials were later determined to have serious toxic side effects or problems in delivery.[2–4] The therapeutic use of most drugs currently on the market (such as metformin and glibenclamide), is limited due to toxic side effects.[5–7] Therefore, the search for the ideal drug to ameliorate high blood glucose levels continues. Chronically elevated blood glucose levels in patients with type II diabetes is caused by excessive glucose production by the liver coupled with decreased glucose uptake and metabolism by muscle, fat, and liver. The gluconeogenesis pathway and rate-limiting enzymes within this pathway such as FBPase have been the focus of efforts to treat diabetes by decreasing glucose production. The prevention of hyperglycemia through control of gluconeogenesis is widely considered to be a viable strategy for the treatment of type II diabetes. FBPase converts fructose 1,6-bisphosphate (FBP) into fructose 6-phosphate and inorganic phosphate; inhibition of this step along the gluconeogenesis pathway has been found to be effective in the control of blood sugar levels in rat models of the pre-diabetic state.[4,8]

In a recent study[5] we synthesized and evaluated a broad group of compounds including substituted pyrazoles, pyrroles, indoles, and carbazoles for inhibition of FBPase in order to identify potential lead compounds that modulate the activity of this enzyme. Based on enzyme inhibition assays and docking studies we identified a small group of leads. These compounds showed similar or better IC_{50} values (3.1, 4.8, 6.1, and 11.9 μM) than that of adenosine monophosphate (AMP), the natural inhibitor of murine FBPase ($IC_{50}=4.0$ μM). Docking programs were used to interpret the experimental results.[5]

In this study we built upon our previous SAR data, and by the use of extended targeted molecular modeling and docking studies, we designed and evaluated a broad variety of new inhibitor candidates. Based on the in silico results, we identified and synthesized five new inhibitor compounds for synthesis. Herein we report two new improved lead compounds for the inhibition of FBPase. We used three of the most commonly applied biochemical modeling programs to carry out parallel docking studies with a broad range of derivatives of the original lead compounds. Although there are three potential binding sites in the FBPase structure to target,[9] according to the results from our previous study as well as other studies, we in-

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vestigated small-molecule binding at the allosteric regulatory site that binds AMP (Figure 1).[5,10]

After analysis of the results and comparison of the docking data with those of the natural inhibitor AMP, as well as taking synthetic approaches into consideration (difficulty, cost, and stability), five compounds were synthesized. The compounds prepared were then tested in FBPase inhibition assays to determine their potency; the results were then compared with that of AMP.

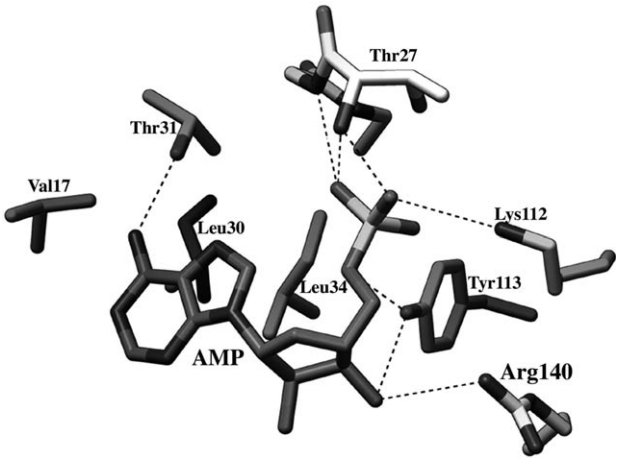


Figure 1. A close-up view of the AMP binding pocket of FBPase (PDB ID: 1KZ8[9]). The image was produced by using POVScript+.[33] The actual position of AMP in the crystallographic structure is shown. The docking programs were tested for accuracy using AMP as previously described.[5]

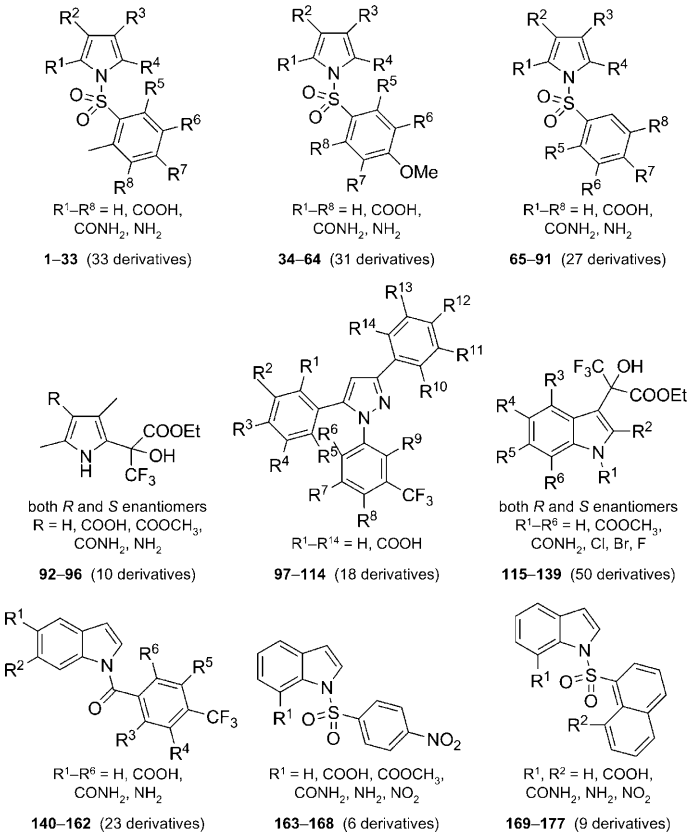


Figure 2. The library of pyrrole-, pyrazole-, and indole-based potential FBPase inhibitor candidates used in this study (see the Supporting Information for individual structures).

Results and Discussion

The small-molecule structures used in this study were generated based on our earlier investigations.[5] The major groups analyzed were indoles, pyrroles, and pyrazoles. The compounds were evaluated virtually by using the most commonly applied biomolecular docking programs such as AutoDock 4, Dock 6, and Surflex 2. Based on the position of chosen ligands in the protein pocket and on the evaluation of functional groups of our protein located next to the ligand, we visually determined where interactions might occur and what types of functional groups would likely improve both theoretical docking scores and experimentally determined IC_{50} values. The most reasonable functional groups were NH_2, CH_2NH_2, COOH, $COOCH_3$, and $CONH_2$. The three lead compounds were systematically modified by retaining their original scaffold and adding hydrophilic or lipophilic substituents of various sizes. We designed and constructed a library of inhibitor candidates (207) to provide a systematic variation of several substituents. A schematic representation of the compounds used in the docking studies is shown in Figure 2, and the complete list is provided in the Supporting Information (figure S1).

The new modified structures were drawn in ChemDraw Ultra 9.0, optimized in Gaussian 98,[11] and tested with the three docking programs mentioned above. The FBPase crystal structure was modified by removing the natural inhibitor AMP, followed by insertion of the ligand in the known allosteric pocket of the enzyme. The complete series of docking results are summarized in table S1 (Supporting Information). Based on the calculated binding energies and binding constants (predicted K_i values in AutoDock) of the small-molecule structures in the three docking programs, we selected the best 13 compounds. For each docking program used, the docking scores of the potential inhibitors (small molecules) were compared with that of AMP. The compounds predicted to bind FBPase with greater than twofold higher affinity than AMP were selected for viewing interactions in the AMP binding pocket by the molecular graphics program Chimera, and were modified as described above. The program X-Score[12,13] was used as a common measurement tool for comparison of performance of the small molecules in the docking programs. The binding parameters (energies, K_i values) of these compounds in the inhibitor–FBPase interaction are listed in Table 1.

As summarized in Table 1, through docking, we identified 13 compounds as candidates for chemical synthesis. The compounds in Table 1 are organized with docking scores from Surflex 2 greater than that of AMP (**142**, (*R*)-**95**, **100**, **106**, **147**, and **176**). Molecules are also organized with Dock 6 docking scores lower than AMP, including **21**, **84E**, **64**, **84**, **160**, and **152**. The compounds selected for synthesis were: 1-[4-(trifluoromethyl)-benzoyl]-1*H*-indole-5-carboxamide (**142**), 1-(α-naphthalen-1-yl-sulfonyl)-7-nitro-1*H*-indole (**176**), 5-(4-carboxyphenyl)-3-phenyl-

Table 1. Docking data for AMP and the 13 best inhibitor candidates.

Compd	Surflex 2 Energy Score	Dock 6 Energy Score	AutoDock 4 Final Docked Energy	X-Score Energy Score
AMP	4.00	−25.66	−15.23	−23.38
142	5.33	−25.19	16.03	−23.47
(*R*)-**95**	5.09	−19.32	−14.26	−16.81
100	4.82	−21.85	−9.17	−22.68
106	4.70	−26.85	−15.70	−22.90
(*S*)-**120**	4.60	−21.54	−12.02	−16.57
147	4.27	−21.41	−11.77	−23.52
21	4.13	−35.68	−15.59	−15.43
176	4.12	−25.76	−15.36	−22.52
84 E	4.02	−34.22	−18.50	−25.20
164	2.91	−33.53	−15.97	−20.26
84	4.43	−31.18	−15.25	−23.84
160	4.15	−29.48	−9.42	−16.04
152	3.47	−29.46	−9.22	−24.32

1-[3-(trifluoromethyl)phenyl]-1*H*-pyrazole (**100**), 1-(4-carbomethoxyphenylsulfonyl)-1*H*-pyrrole (**84 E**), and 1-(4-carboxyphenylsulfonyl)-1*H*-pyrrole (**84**). Compounds **142** and **176** could be synthesized by a base-assisted N acylation of the corresponding commercially available indole derivatives with the appropriate acid chlorides, and the two were produced by using essentially the same procedure. The pyrazole derivative **100** was synthesized by a domino cyclization-aromatization process with **100 E**, and then it was hydrolyzed by a solution of potassium hydroxide in ethanol. Compound **84 E** was prepared by a method we reported earlier, a K-10 montmorillonite-catalyzed cyclization process;[14] the ester was hydrolyzed to furnish **84** by the same method mentioned above for the conversion of **100 E** into **100**. Despite moderate yields in certain cases, we were able to obtain the products, and tested their activity in FBPase inhibition. Notably, we attempted the synthesis of other compounds that were also identified as reasonable candidates. In some cases, despite successful synthesis and proper identification by mass spectrometry, the stability of the product was not satisfactory; in most cases the half-life of these compounds was less than 2–3 h. These stability problems limited the number of inhibitor candidates for biological assays to five.

Because AMP is the natural inhibitor of FBPase, any new proposed inhibitor for this enzyme was not only docked into the allosteric AMP binding site, but was also compared with AMP by determination of its IC_{50} value. Based on earlier studies, we determined the biological activity of the selected compounds in a murine FBPase inhibition assay. For direct comparison, the potency of AMP was also determined under identical conditions, and the IC_{50} value of AMP was determined to be 4.0 μM. For **84 E**, **84**, **100**, **142**, and **176**, the respective IC_{50} values were found to be 32 nM, 135 nM, 575 nM, 991 nM, and 1.34 μM. It appears that the computer-based systematic inhibitor design resulted in compounds that are more potent than AMP by approximately two orders of magnitude. Our data clearly indicate that not only the *N*-sulfonylpyrroles **84** and **84 E**, but also the pyrazole derivative **100** gave significantly lower IC_{50} values than those of the indole derivatives **142** and **176**. We were therefore able to narrow down the basic scaffold in our second-generation lead compounds to the triarylpyrazoles and, most importantly, to the substituted *N*-sulfonylpyrroles (Scheme 1). Importantly, the activity of our new inhibitors, especially **84 E** (IC_{50} = 32 nM) is virtually the same as that of the best currently known inhibitor compounds.[2–4]

Docking studies and visual examination of the results using Chimera suggested that similar interactions of the molecules occur in the AMP binding pocket relative to the natural inhibitor AMP, as reported previously.[5] More specifically, both **142** and **176** showed hydrogen bonding and electrostatic interactions with Lys 112, Tyr 113, and Arg 140, as well as enhanced (short-distance) hydrophobic interactions with Leu 30 and Val 160 compared with AMP. Compound **100** also showed shorter distances than the phosphate group of AMP in hydrogen bonding of its carboxylic acid moiety with Tyr 113, Lys 112, Thr 27, and Arg 140. The fluorine atoms are buried in the binding pocket and interact with Thr 31 and the backbone of Gly 21 (Figure 3 A). The sulfate group oxygen atoms of compound **84** interact with Arg 140 and Tyr 113 and the backbone of Thr 31 (Figure 3 B). Compound **84 E** exhibits enhanced hydrophobic interactions with Val 160, Leu 30, and Leu 34 of FBPase; its sulfate group oxygen atoms interact with Arg 140 and Tyr 113, as well as the backbone of Thr 31 (Figure 3 C). The most striking difference from AMP was the number of new interactions established in the hydrophobic interior of the AMP binding pocket (Figure 3).

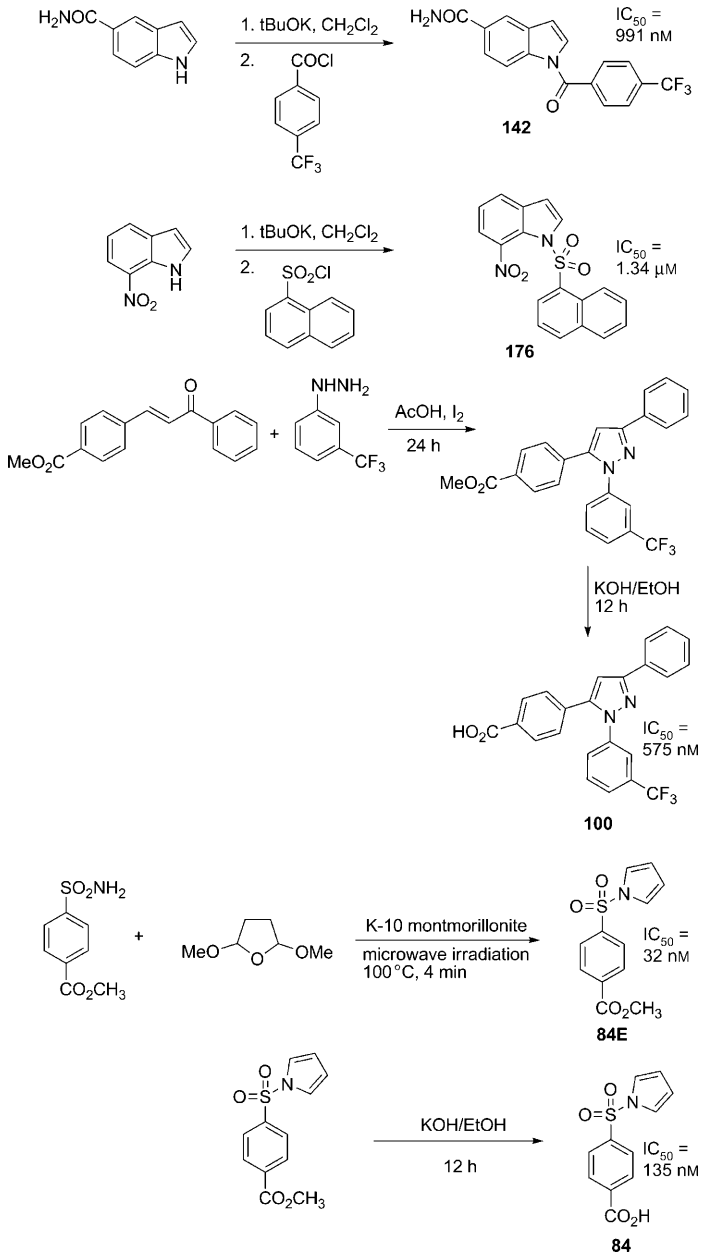
Conclusion

In summary, we applied computer-based drug design using docking programs and graphics-display programs to systematically select molecules for synthesis, testing, and analysis in FBPase inhibition. The synthesized compounds were tested for their biological potency by an in vitro enzyme inhibition assay. The biological response to the new compounds is approximately two orders of magnitude better than that generated by the natural inhibitor AMP. These results highlight two *N*-sulfonylpyrroles and a triarylpyrazole as our second-generation lead compounds, with potency similar to that of the best currently known compounds.

Experimental Section

Design of inhibitor candidates

The small-molecule structures used in this study were generated as described below. Three lead structures were systematically changed by retaining their original scaffold and adding substituents of various character. These major groups were indoles, pyrroles, and pyrazoles. Thus a wide range of derivatives was constructed to provide a systematic variation of the substituents for docking studies. The following method was used to propose the substituents: the FBPase crystallographic structure was modified by removing the natural inhibitor AMP, and then replacing it with the ligand in question by docking to the allosteric AMP binding site.



Scheme 1. Synthesis and biological potency of the second-generation lead compounds.

Docking studies

The 3D crystal structure coordinates were downloaded from the RCSB Protein Data Bank (PDB).[15] The ligand was removed from the binding pocket of interest. The energy-minimized ligand with full charges was loaded into the program PRODRG.[16] The format used was mol2 for Dock 6[17] and Surflex 2,[18] whereas PDBQ format was used for AutoDock 4.[19] The molecules were drawn in GaussView 2.1,[11] energy-minimized (HF/STO-3G), and converted into PDBQ then mol2 format (ChemDraw Ultra 9.0 and Dundee PRODRG). The structure of FBPase (PDB ID: 1FTA) was obtained from the RCSB PDB.

Using Surflex-Dock 2.0, a conformational search of rotatable bonds was performed, and these conformations were aligned to the protein (protomol); the binding energy was then determined in log format. AutoDock 4 was used to dock the ligand into a set of grids describing the target protein, and this provided estimated inhibition constants in log format. Dock 6 was used to generate a set of overlapping spheres that fill the active site. To orient a ligand within the active site, some of the sphere centers were "matched" with ligand atoms. Approximations were made to the usual molecular mechanics attractive and dispersive terms for use on a grid. The program presented energy scores in log format. X-Score[12,13] was used to compare binding energies by analyzing binding strength between the protein and ligand, as determined by electrostatic, hydrophilic, and hydrophobic interactions.

Synthesis of inhibitor candidates

All chemicals and solvents were purchased from Aldrich. The inhibitor candidates were synthesized based on published methods.[20–24] In all cases, compounds were purified by preparative thin-layer chromatography (TLC). Identification and determination of purity were carried out by gas chromatography–mass spectrometry with an Agilent 6850-5973N GC–MS system (70 eV electron impact ionization, 30 m long DB-5 type column) and NMR spectroscopy (^{1}H, ^{13}C, and ^{19}F when applicable) using a 300 MHz superconducting Varian NMR spec-

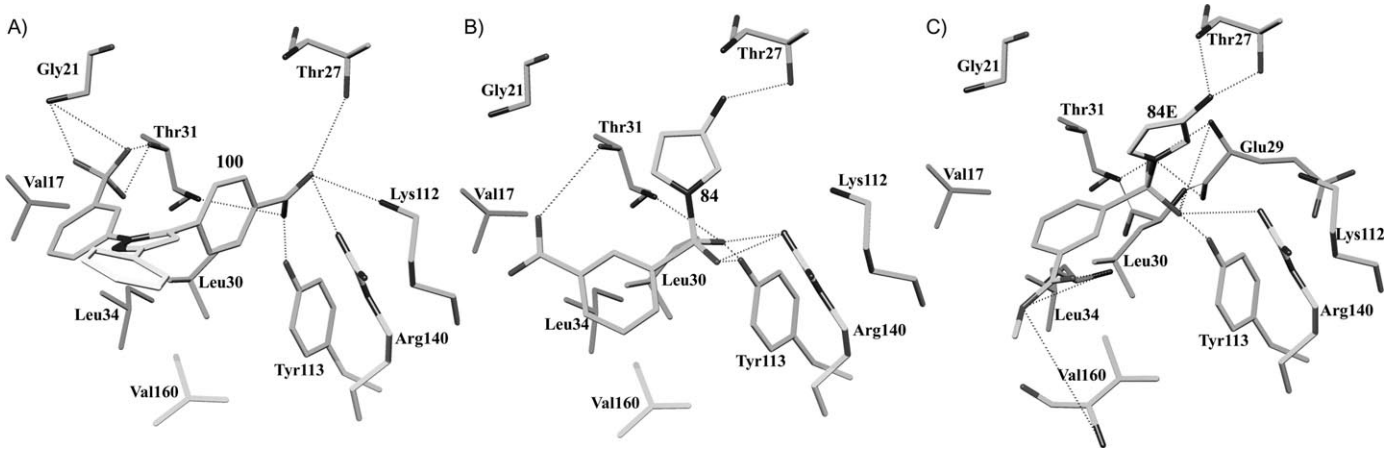


Figure 3. AMP binding pocket of human FBPase (PDB ID: 1FTA)[34] with the docking results of the three best inhibitors: A) **100**, B) **84**, and C) **84E**. This image was generated with the program Chimera.[35]

trometer, with [D_6]DMSO or $CDCl_3$ as solvents and $(CH_3)_4Si$ as internal standard. Coupling constants are given in Hz. The purity of the compounds exceeded 95% (GC).

1-(4-Carbomethoxyphenylsulfonyl)-1*H*-pyrrole (84E): 4-Carbomethoxyphenylsulfonamide (1 mmol) and 2,5-dimethoxytetrahydrofuran (1.5 mmol) were mixed in Et_2O (3 mL) in a round-bottom flask. K-10 montmorillonite (500 mg) was then added. After stirring for 5 min, the solvent was evaporated to produce a dry mixture of reactants adsorbed at the catalyst surface. The dry mixture was transferred to a reaction tube and irradiated in a focused microwave reactor (CEM Discover Benchmate) at 100 °C. The reaction temperature was determined and maintained by a built-in infrared temperature detector–controller. After satisfactory conversion, Et_2O (10 mL) was added to the cold mixture, and the product was separated from the catalyst by filtration. The product was isolated as colorless crystals and purified by flash chromatography to give 198 mg **84E** (75% yield): mp: 115–117 °C (dec.); ^{1}H NMR (300.126 MHz, $CDCl_3$): δ = 8.12 (d, J = 8.1 Hz, 2H), 7.91 (d, J = 8.1 Hz, 2H), 7.16 (d, J = 4.8 Hz, 2H), 6.31 ppm (d, J = 4.8 Hz, 2H); ^{13}C NMR (74.467 MHz, $CDCl_3$): δ = 165.15, 142.59, 134.76, 130.34, 127.73, 127.37, 120.81, 114.18, 52.72 ppm.

1-(4-Carboxyphenylsulfonyl)-1*H*-pyrrole (84): 4-Carbomethoxyphenylsulfonamide (120 mg) was dissolved in EtOH (0.1 mL). KOH (8 M, in 0.1 mL EtOH) was then added to the solution, which was stirred for 12 h at room temperature. After satisfactory conversion, the solution was acidified to pH 1 with concd HCl. A white precipitate formed which was washed (H_2O, 15 mL) and dried. The crude product was purified by flash chromatography to give 98 mg **84** (89% yield). Colorless crystals: mp: 225–227 °C (dec.); ^{1}H NMR (300.126 MHz, [D_6]DMSO): δ = 8.14 (d, J = 8.7 Hz, 2H), 8.08 (d, J = 8.7 Hz, 2H), 7.39 (t, J = 2.1 Hz, 2H), 6.40 (d, J = 2.1 Hz, 2H), 3.39 ppm (br s, 1H); ^{13}C NMR (74.467 MHz, [D_6]DMSO): δ = 166.38, 142.04, 136.56, 131.17, 127.69, 121.82, 115.02 ppm.

5-(4-Carbomethoxyphenyl)-3-phenyl-1-[3-(trifluoromethyl)-phenyl]-1*H*-pyrazole (100E): (*E*)-methyl-4-(3-oxo-3-phenylprop-1-enyl)benzoate (266 mg, 1 mmol) and 3-trifluoromethyphenylhydrazine (157 μL, 1.2 mmol) were dissolved in AcOH (5 mL), followed by the addition of I_2 (253 mg). The mixture was stirred for 24 h at room temperature and then quenched with a saturated solution of Na_2CO_3 (5 mL). The product was extracted with Et_2O (3×15 mL). The combined organic extracts were washed with a saturated solution of $Na_2S_2O_3$ (10 mL), dried over anhydrous Na_2SO_4, and the solvent was removed under vacuum. The crude product was purified by flash chromatography to yield 320 mg **100E** (76% yield). Colorless crystals: mp: 189–192 °C (dec.); ^{1}H NMR (300.126 MHz, [D_6]DMSO): δ = 8.03 (d, J = 8.4 Hz, 2H), 7.92 (d, J = 8.4 Hz, 2H), 7.78 (br s, 1H), 7.59 (m, 1H), 7.35–7.46 (m, 7H), 6.92 (s, 1H), 3.94 ppm (s, 3H); ^{13}C NMR (74.467 MHz, $CDCl_3$): δ = 172.30, 171.10, 152.82, 143.50, 134.35, 132.31, 130.15, 129.94, 129.51, 128.77, 128.62, 128.46, 128.05, 125.85, 124.21, 122.06, 117.09, 105.59 ppm; ^{19}F NMR (300 MHz, $CDCl_3$): δ = −63.10 ppm; MS (70 eV) m/z (I_{rel}): 422 (100%) $[M]^+$, 423 (26%) $[M]^+$, 391 (23%), 392 (6%).

5-(4-Carboxyphenyl)-3-phenyl-1-[3-(trifluoromethyl)phenyl]-1*H*-pyrazole (100): Compound **100E** (175 mg) was dissolved in EtOH (0.2 mL). KOH (8 M, 76 μL in EtOH) was then added to the solution. The mixture was stirred for 12 h at room temperature and then acidified to pH 1 with concd HCl. The precipitate was filtered off, washed with H_2O (5 mL), and dried for 12 h. The crude product was purified by preparative TLC to give 145 mg **100** (87% yield). White crystals: mp: 215–217 °C (dec.); ^{1}H NMR (300.126 MHz, $CDCl_3$): δ = 8.09 (d, J = 8.4 Hz, 2H), 7.93 (d, J = 8.4 Hz, 2H), 7.78 (br s, 1H), 7.59 (m, 1H), 7.38–7.49 (m, 7H), 6.93 ppm (s, 1H); ^{13}C NMR (74.467 MHz, $CDCl_3$): δ = 215.85, 171.40, 152.88, 143.31, 140.09, 135.19, 132.23, 130.54, 129.56, 129.24, 128.78, 128.70, 128.51, 128.08, 125.86, 124.30, 122.08, 106.64 ppm; ^{19}F NMR (300 MHz, $CDCl_3$): δ = −63.07 ppm.

1-[4-(trifluoromethyl)benzoyl]-1*H*-indole-5-carboxamide (142): Indole-5-carboxamide (1 mmol, 160 mg) was dissolved in CH_2Cl_2 (1 mL), and *t*BuOK (1.1 mmol, 123 mg) was carefully added at room temperature. The bright-orange solution was stirred for 30 min, and then 4-trifluoromethylbenzoyl chloride (1.2 mmol, 178 mg) was added. The reaction mixture was allowed to react under continuous stirring for 12 h. The reaction was then poured into distilled H_2O (2 mL), and the product was extracted with CH_2Cl_2. The combined extracts were dried over Na_2SO_4, and the solvent was removed under vacuum. The crude product was purified by preparative TLC to give 22 mg **142** (7% yield). ^{1}H NMR (300.126 MHz, [D_6]DMSO); δ = 8.68 (d, J = 8.4 Hz, 1H), 8.53 (m, 3H), 8.26 (d, J = 8.7 Hz, 2H), 7.78 (d, J = 3.9 Hz, 1H), 7.74 (s, 2H, NH_2), 7.22 ppm (d, J = 3.3 Hz, 1H); ^{13}C NMR (74.467 MHz, [D_6]DMSO): δ = 169.51, 168.09, 138.20, 138.02, 137.62; 131.14, 130.76, 130.43, 129.84, 127.37, 126.48, 126.21, 125.01, 121.58, 121.41, 121.13, 116.14, 112.37, 111.66, 110.15 ppm; ^{19}F NMR (282.4 MHz, [D_6]DMSO): δ = −60.87 ppm; MS (70 eV) for $C_{17}H_{11}N_2O_2F_3$: m/z (%): 332 $[M]^+$ (100), 313 (17), 173 (85), 145 (40).

1-(α-naphthalen-1-ylsulfonyl)-7-nitro-1*H*-indole (176): *t*BuOK (1.1 mmol, 123 mg) was carefully added to a solution of 7-nitroindole (1 mmol, 162 mg) in CH_2Cl_2 at room temperature. The bright-orange solution was stirred for 30 min, and α-naphthylsulfonyl chloride (1.2 mmol, 272 mg) was then added. The reaction mixture was allowed to react under continuous stirring for 12 h. The reaction was then poured into distilled H_2O (2 mL), and the product was extracted with CH_2Cl_2. The combined extracts were dried over Na_2SO_4, and the solvent was removed under vacuum. The crude product was purified by preparative TLC to give 215 mg **176** (60% yield). ^{1}H NMR (300.126 MHz, $CDCl_3$): δ = 8.42 (m, 2H), 8.13 (d, J = 8.1 H, 1H), 7.97 (m, 2H), 7.84 (d, J = 7.8 Hz, 1H), 7.75 (d, J = 8.1 H, 1H), 7.68 (d, J = 3.9 Hz, 1H), 7.61 (m, 2H), 7.57 (dd, J = 7.5, 8.1 Hz, 1H), 7.38 (dd, J = 8.1, 7.8 Hz, 1H), 6.82 ppm (d, J = 3.9 Hz, 1H); ^{13}C NMR (74.467 MHz, $CDCl_3$): δ = 135.12, 134.08, 130.71, 129.06, 128.80, 127.88, 127.63, 127.28, 126.43, 125.85, 123.94, 123.81, 123.36, 120.88, 116.75, 108.92 ppm; MS (70 eV) for $C_{18}H_{12}N_2O_4S$: m/z (%): 352 $[M]^+$ (92), 241 (18), 191 (55), 127 (100).

FBPase assays

Acetone-precipitated murine liver homogenates were resuspended in deionized H_2O, sterile filtered, and then purified and dialyzed against 50 mM Tris buffer (pH 7.5) for three changes over 48 h at 4 °C. The crude protein extract was then run over a gel filtration column (G25–150), and the fractions were analyzed by Bradford assay and SDS-PAGE. Fractions containing FBPase activity were pooled and re-dialyzed in 50 mM Tris (pH 7.5), loaded on a Matrix Gel Blue Affinity column (Cibacron Blue 3GA dye coupled to cross-linked 5% agarose), and eluted in the presence of 1 mM FBP. Fractions were tested for activity, pooled, and re-dialyzed in 50 mM Tris (pH 7.5). The final concentration of wild-type enzyme was determined by absorption at λ 280 nm (A_{280})[25] and compared with the Bio-Rad version of the Bradford dye-binding assay.[26] Enzyme purity was verified by SDS-PAGE[27] and non-denaturing PAGE.[28,29]

The FBPase high-throughput screen is based on measurement of phosphatase activity by colorimetric assay using an ammonium molybdate Malachite Green reagent.[30,31] The reagent was pre-

pared with a 1:3 ratio of 4.2% ammonium molybdate and 0.05% malachite green oxalate. Specific activity was calculated from calibration curves, 2–20 μM, that were made with a standard KH_2PO_4 solution. The reaction was carried out at 37 °C for 3 min, and then stopped by quenching the samples with the addition of 1 mL of the colorimetric phosphate assay dye reagent to each sample. The specific activity was estimated by using the A_{660} value to determine product formation (in μmol) using the standard KH_2PO_4 solution as a quantitative measure of inorganic phosphate present in the reaction mixture.

To determine more detailed kinetic parameters such as K_i values, the total volume of the assay mixture was increased to 200 μL in order to minimize experimental error. The amount of enzyme was adjusted to keep the A_{660} value between 0.1 and 0.5, in order to keep the percentage of product <20% of total substrate.[32]

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Keywords: docking · heterocycles · indoles · inhibitors · N substitution

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