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Design and synthesis of thiamine analogues to study their binding to the ECF transporter for thiamine in bacteria†‡

 L. Monjas,§^a L. J. Y. M. Swier,§^b A. R. de Voogd,^a R. C. Oudshoorn,^b
A. K. H. Hirsch*^a and D. J. Slotboom*^b

Energy-coupling factor (ECF) transporters mediate the uptake of vitamins in bacteria. Given that these ECF transporters are not present in eukaryotic cells, they represent an interesting target for the development of novel antibiotics. Here, we present the design and synthesis of compounds that bind to ThiT, the substrate-binding domain of the ECF transporter for thiamine from *Lactococcus lactis*. We modified the methyl substituent of the pyrimidine ring of thiamine, in order to evaluate its contribution to the binding affinity. Our results indicate that as long as a hydrophobic substituent is maintained, the high binding affinity is almost unchanged, opening up opportunities for the design of selective compounds.

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Introduction

Energy-coupling factor (ECF) transporters form a distinct type of importers in the family of ATP-binding cassette (ABC) transporters and mediate the uptake of essential nutrients, such as vitamins, cofactors and transition metal ions, in archaea and bacteria.^{1,2a} ECF transporters are predominantly found in Gram-positive bacteria, including many human pathogens. A significant fraction of these human pathogens lack enzymes for the *de novo* synthesis of many vitamins and cofactors transported by ECF transporters, which makes the presence of these transporters essential for those pathogens. Given that eukaryotic cells do not make use of ECF transporters, but instead use unrelated secondary transport systems,^{2b} ECF transporters represent an attractive target for the development of novel antibiotics.

ECF transporters consist of four subunits: two identical or highly similar, cytoplasmic nucleotide-binding domains known as EcfA and EcfA', and two transmembrane domains called EcfT and S-component (Fig. 1A). EcfA and EcfA' bind and hydrolyze ATP to fuel substrate transport. These two domains interact with EcfT, forming together an energizing module, which interacts with the fourth subunit, the

S-component. This S-component functions as the substrate-binding domain, which binds its corresponding substrate with high affinity.

Studies on ECF transporters started only recently and the mechanism of transport is poorly understood. Five crystal structures of substrate-bound S-components,^{3,5–8} as well as three crystal structures of full transporters,^{9–11} have been reported over the past five years and have provided first insights into the structural basis of transport. Among these crystal structures, the co-crystal structure of ThiT from *Lactococcus lactis*, in complex with thiamine (1, vitamin B₁) has been solved at a resolution of 2.0 Å (PDB ID: 3RLB, Fig. 1B).³ ThiT is the S-component in the ECF transporter for

Fig. 1 (A) Schematic representation of an ECF transporter. The two nucleotide-binding domains EcfA and EcfA' are shown in two shades of red, the EcfT in blue and the S-component in gray. (B) Secondary-structure representation of ThiT from *Lactococcus lactis* in complex with thiamine (PDB ID: 3RLB).³ ThiT is shown in gray, and thiamine is shown in sticks using the following color code: C: green, O: red, N: blue, S: yellow. This color code for the oxygen, nitrogen, and sulfur atoms is maintained throughout the article. The figure and other figures of this type throughout the article were generated with PyMOL version 1.7.6.⁴

^a Stratingh Institute for Chemistry, University of Groningen, Nijenborgh 7, 9747 AG Groningen, The Netherlands. E-mail: a.k.h.hirsch@rug.nl; Tel: +31 50 363 4275

^b Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands. E-mail: d.j.slotboom@rug.nl; Tel: +31 50 363 4187

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§ These authors contributed equally to this work.

thiamine (**1**) and binds this substrate with sub-nanomolar affinity (Fig. 2).

In our previous work, we described the first series of thiamine derivatives that bind to ThiT.¹³ These small-molecule binders were designed by modifying the thiazolium ring and the hydroxyethyl side chain of thiamine (**1**), and they show binding affinities for ThiT ranging over three orders of magnitude ($K_D = 0.18$ –528 nM), allowing us to understand how different functional groups contribute to the high binding affinity. Here, we report new thiamine derivatives, in which we modified the methyl substituent of the pyrimidine ring of deazathiamine (**2**, Fig. 2). This methyl group occupies a small, hydrophobic cavity in the substrate-binding pocket with a diameter of 4.3–6.5 Å, and the residues lining this cavity are conserved in different ThiT homologues.^{3,12} Our aim is to shed light on the mechanism of substrate binding and transport of ECF transporters by using modified substrates, which may lead to new antibiotics that could block vitamin transport in pathogenic bacteria that rely on these transporters to survive.

Results and discussion

Design of small-molecule binders

We designed small-molecule binders for ThiT using the co-crystal structure of ThiT in complex with its natural substrate thiamine (**1**; PDB ID: 3RLB).³ Thiamine interacts with many residues lining the substrate-binding pocket (Fig. 3A): the amino group forms two hydrogen bonds with Glu84 and His125, and the two pyrimidine ring nitrogen atoms form hydrogen bonds with Asn151 and, *via* an ordered water molecule, with Tyr146. The pyrimidine ring is also involved in a π - π stacking interaction with Trp133, and the thiazolium ring of thiamine forms a π -sandwich with Trp34 and His125. The positively charged nitrogen atom in the thiazolium ring is involved in an electrostatic interaction with Glu84. Finally, the hydroxyl group interacts *via* a hydrogen bond with Tyr85.

In this study, we probed the role of the methyl substituent of the pyrimidine ring, which occupies a small lipophilic pocket, interacting with residues Gly129, Trp133, Tyr146, Ser147 and Val150 (Fig. 3B). The first four residues are well-conserved in different ThiT homologues,³ and mutagenesis studies have shown that Gly129, Trp133 and Tyr146 are necessary to maintain the high binding affinity for thiamine (**1**).¹² As mentioned before, Trp133 and Tyr146 are involved in π - π stacking and hydrogen-bonding interactions, respectively, with the pyrimidine ring of thiamine (**1**). In the case of

Fig. 3 (A) The binding mode of thiamine in the binding pocket of ThiT (PDB ID: 3RLB).³ The substrate-binding residues of ThiT and thiamine are shown in stick representation, with the carbon atoms of ThiT and thiamine in gray and green, respectively. The black dashed lines indicate hydrogen bonds below 3.6 Å, and the red sphere represents an ordered water molecule. (B) Stereo view with surface representation of the residues lining the small, hydrophobic cavity occupied by the methyl group of thiamine, Val150 is positioned behind the cavity.

Gly129, it only has hydrophobic and van der Waals interactions with the methyl substituent of thiamine (**1**). The Gly129Ala mutation shows a significant decrease in affinity ($K_D = 33.9 \pm 3.9$ nM), whereas the Gly129Val mutation abolishes binding, most probably due to steric clashes with the ligand.¹² Based on these findings, we wanted to probe possible extensions on the pyrimidine ring of thiamine that would interact with this part of the substrate-binding pocket.

We used the software MOLOC for molecular modeling,¹⁴ and the LeadIT suite to predict the binding mode (FlexX docking module)¹⁵ and to estimate the Gibbs free energy of binding (HYDE scoring function)^{16,17} of the designed molecules. Because the binding mode of deazathiamine (**2**) is almost the same as that of thiamine (**1**)¹³ (except for the electrostatic interaction of the positively charged nitrogen atom with Glu84, which is lost in the case of **2** given that it is a neutral molecule), we designed five new compounds with a thiophene ring instead of a thiazolium ring to make these compounds synthetically more accessible. We substituted the methyl group of the pyrimidine ring for groups with different properties (Fig. 4): a hydrogen atom (**3**) or an amino (**4**), trifluoromethyl (**5**), ethyl (**6**) and isopropyl (**7**) group.

Fig. 2 The chemical structures of thiamine (**1**) and deazathiamine (**2**), and the binding affinities of ThiT for these compounds.^{12,13}

Fig. 4 The chemical structure of the designed deazathiamine derivatives.

Removal of the methyl group to afford the unsubstituted derivative **3** leads to a decrease in the predicted binding affinity (Table 1, $\Delta G_{\text{est}} = -44 \text{ kJ mol}^{-1}$) in comparison with our reference compound **2** ($K_D = 4.23 \pm 1.69 \text{ nM}$, $\Delta G_{\text{exp}} = -48 \text{ kJ mol}^{-1}$). The same trend is predicted when we introduce an amino group (**4**); in this case, the loss of affinity would be even bigger ($\Delta G_{\text{est}} = -38 \text{ kJ mol}^{-1}$). Compound **5**, with a $-\text{CF}_3$ group, which is more lipophilic than $-\text{CH}_3$, would improve the binding affinity of deazathiamine (**2**), with $\Delta G_{\text{est}} = -56 \text{ kJ mol}^{-1}$. Alkyl groups such as ethyl (**6**) and isopropyl (**7**) groups would also bind with higher affinity compared to deazathiamine (**2**), with $\Delta G_{\text{est}} = -57$ and -50 kJ mol^{-1} , respectively. According to these estimates, there is a clear trend: a polar group (NH_2 , compound **4**) would have a weaker binding affinity than a hydrogen atom (compound **3**), and alkyl groups would lead to stronger binding. When we tried to model groups bigger than isopropyl, the repulsions with residues Gly129, Tyr146 and Val150 were too strong, and this would force the pyrimidine ring to adopt a different pose in the binding pocket, weakening the hydrogen bonds with Glu84, His125 and Asn151, so isopropyl appears to be the biggest substituent that ThiT can accommodate in this part of the substrate-binding pocket.

Synthesis of small-molecule binders

We synthesized compounds **3–7** by modifying the last step of our previously reported synthesis of compound **2** (Scheme 1).¹³ Enamine **8** was synthesized in four steps from commercially available 2,3,5-tribromo-4-methylthiophene. The aminopyrimidine ring was formed by the reaction of **8** with guanidine (for compound **4**) or different amidines (for compounds **3** and **5–7**), under basic conditions, at reflux for 1–5 days, affording the desired products in 27–62% yield.

Binding-affinity determination

We determined the binding affinities (K_D values) of ThiT for the synthesized compounds by using an intrinsic protein fluorescence titration assay¹² (compounds **3** and **5–7**) or isothermal ti-

Scheme 1 The synthesis of compounds **3–7**. Reagents and conditions: (a) $^t\text{BuLi}$, Et_2O , -78°C , 5 min, 93%; (b) 1: $^t\text{BuLi}$, Et_2O , -78°C , 1 h, 2: ethylene oxide, $\text{BF}_3\cdot\text{Et}_2\text{O}$, -78°C to room temperature, 2 h, 68%; (c) 1: $^i\text{PrMgCl}$, THF, 0°C , 10 min, 2: $^t\text{BuLi}$, -78 to -40°C , 1 h, 3: DMF, -78°C to room temperature, 16 h, 65%; (d) 3-anilinopropionitrile, NaOEt , DMSO, microwave, 70°C , 30 min, 71%; (e) $\text{RC}(=\text{NH})\text{NH}_2$, NaOMe (or NaOEt), MeOH (or EtOH), reflux, 1–5 d, 27–62%.

tration calorimetry (ITC) (compound **4**). In ITC, it is hard to determine accurate K_D values in the low nanomolar range, since the transition between binding events and saturation of the protein with a ligand is covered by only a few data points. In the intrinsic protein fluorescence assay, this transition is covered by far more data points, which allows the use of this assay to determine accurate K_D values in the nanomolar range. However, for compound **4**, we were unable to use the intrinsic protein fluorescence assay because of a lack of intrinsic fluorescence quenching upon ligand addition.

As predicted by modeling and docking, the binding affinities of compounds **3** ($\text{R} = \text{H}$) and **4** ($\text{R} = \text{NH}_2$) are lower than the binding affinity determined for deazathiamine (**2**), with K_D values in the micromolar range (Table 1). Compounds **5–7**, which bear alkyl groups, have binding affinities in the same range as that for compound **2**, with the K_D of **6** ($\text{R} = \text{Et}$) being slightly, but not significantly, lower than those of **5** ($\text{R} = \text{CF}_3$) and **7** ($\text{R} = ^i\text{Pr}$), in agreement with the predictions of docking and scoring using the scoring function HYDE.

As a preliminary evaluation of selectivity, we have docked the natural substrate or cofactor and the new thiamine derivatives into the available crystal structures of human thiamine-binding proteins (Table 2). Although these proteins are not involved in the transport of thiamine across cell membranes, the docking studies provide an indication of the structural variation among thiamine-binding proteins. For thiamine pyrophosphokinase 1 (PDB ID: 3S4Y), the new thiamine derivatives **2–7** would also bind, given that their ΔG_{est} values are comparable to those of the natural substrate TDP. Compound **4**, featuring an amino group, would be a weak binder. For transketolase (PDB ID: 3OOY), compounds **2** and **6** would bind as strongly as the natural substrate. Compound **7** is predicted not to bind and should therefore be selective for ThiT. For thiamine triphosphatase (PDB ID: 3TVL),¹⁸ only compound **5** with a trifluoromethyl substituent is predicted to bind with a predicted affinity comparable to that of the natural substrate/cofactor. For branched-chain α -ketoacid

Table 1 The binding affinities of ThiT for thiamine and the small-molecule derivatives, with the errors indicating the standard deviations, together with the experimental and estimated Gibbs free energy of binding (ΔG). The latter is based on the scoring function HYDE^{16,17}

Compound	$K_D \pm \text{S.D. (nM)}$	$\Delta G_{\text{exp}} (\text{kJ mol}^{-1})$	$\Delta G_{\text{est}} (\text{kJ mol}^{-1})$
1 (ref. 12)	0.122 ± 0.013	−57	−53
2 (ref. 13)	4.23 ± 1.69	−48	−52
3	1025 ± 187^a	−34	−44
4	2917 ± 420^a	−32	−38
5	8.48 ± 5.15^a	−46	−56
6	5.04 ± 0.92^b	−47	−57
7	13.58 ± 6.87^c	−45	−50

^a The error represents the standard deviation obtained from three experiments. ^b The error represents the standard deviation obtained from five experiments. ^c The error represents the standard deviation obtained from four experiments.

Table 2 Estimated Gibbs free energy of binding (ΔG_{est}) of thiamine derivatives for various human thiamine-binding proteins based on the scoring function HYDE^{16,17}

Compound	PDB ID			
	3S4Y	3O0Y	3TVL	1U5B
	ΔG_{est} (kJ mol ⁻¹)			
Natural substrate/cofactor	-33 ^a	-27 ^a	-31 ^b	-38 ^a
2	-32	-27	-19	-30
3	-30	-19	-17	-24
4	-24	-13	-13	-20
5	-34	-23	-28	-30
6	-39	-31	-15	-28
7	-37	-3	-18	-27

PDB IDs = 3S4Y: human thiamine pyrophosphokinase 1, 3O0Y: human transketolase, 3TVL: human thiamine triphosphatase, 1U5B: human branched-chain α -ketoacid dehydrogenase. ^a Thiamine diphosphate (TDP). ^b Thiamine triphosphate (TTP).

dehydrogenase (PDB ID: 1U5B),¹⁹ compounds 2–7 are much weaker binders than the natural substrate. Upon subjecting the crystal structure of pyruvate dehydrogenase (PDB ID: 3EXE)²⁰ to the same analysis, no reasonable docking run could be performed using the LeadIT suite due to substantial intermolecular clashes. In summary, our docking study predicts compound 7 to be selective (*i.e.*, it is a strong binder for ThiT, whilst being predicted to be a weak binder for all other proteins, except for thiamine pyrophosphokinase 1).

Conclusions

We have shown that the methyl group of the pyrimidine ring of thiamine can be modified, maintaining the high binding affinity. The binding affinity of lipophilic groups (CF₃, Et, ⁱPr) is higher than that of more polar groups (NH₂) or just a hydrogen atom. Steric effects are less pronounced than expected, with minor differences in binding affinity for the derivatives featuring -CF₃, -Et, or -ⁱPr groups. Apparently, the hydrophobic cavity that accommodates these groups is more flexible than expected.

Although the K_D values of the new molecules are not significantly better than that of our reference compound deaza-thiamine (2), we have shown that the methyl group can be substituted for different groups (CF₃, Et, ⁱPr) without substantial loss in affinity, which can be useful in future design of thiamine derivatives. This will allow us to design compounds with improved selectivity for ECF transporters over human thiamine transporters and thiamine-dependent enzymes, and with enhanced metabolic stability as CF₃ is metabolically more stable than CH₃.

Experimental part

Modeling and docking

The co-crystal structure of ThiT in complex with thiamine (PDB ID: 3RLB)³ was used. The program MOLOC,¹⁴ which has the MAB force field implemented, was used for modeling, keeping

the coordinates of the protein and the water molecule HOH196 fixed. Subsequently, the designed molecules were docked into the binding pocket of ThiT using the FlexX docking module of the LeadIT suite.¹⁵ For docking, the binding site of ThiT was restricted to 8.0 Å around the co-crystallized thiamine molecule, and the 30 top-scored solutions were retained and subsequently post-scored with the scoring function HYDE.^{16,17} Poses with significant inter- or intramolecular clash terms or unfavorable conformations were excluded after careful visualization, and the remaining solutions were ranked according to their Gibbs free energy of binding.

For docking of human thiamine-binding proteins, we selected the PDB files of wild-type proteins with the best resolution, with the exception of transketolase, given that the PDB ID: 3MOS, although having better resolution than EOOY, gave high intermolecular clashes using the LeadIT suite. Docking was performed following the same procedure as that for ThiT, and the binding pockets were prepared as follows: 3S4Y: chains A and B, binding pocket restricted to 10 Å around the co-crystallized TDP, Ca²⁺ included; 3O0Y: chains A and B, binding pocket restricted to 10 Å around the co-crystallized TDP, Ca²⁺ included; 3TVL: chain A, binding pocket restricted to a 10 Å sphere centred on the phosphorus atom of the alpha-phosphate group of the co-crystallized triphosphate, Mn²⁺ included; 1U5B: chain A, binding pocket restricted to 10 Å around the co-crystallized TDP, Mn²⁺ included.

Synthesis of small-molecule binders

General methods. All reagents were purchased from Sigma-Aldrich, Acros Organics or TCI Europe, and were used without further purification unless noted otherwise. All solvents were reagent-grade, and if necessary, dried and distilled prior to use. All reactions were carried out under a nitrogen atmosphere using dried glassware. Reactions were monitored by thin-layer chromatography (TLC) on silica gel-coated aluminium foils (silica gel 60/Kieselguhr F254, Merck). Flash column chromatography was performed on silica gels (SiliCycle 40–63 µm). Melting points were determined on a Büchi B-545 apparatus. NMR spectra were recorded on a Varian AMX400 spectrometer at 25 °C. Chemical shifts (δ) are reported in ppm relative to the residual solvent peak. Splitting patterns are indicated as (s) singlet, (d) doublet, (t) triplet, (q) quartet, (sept) septet, (m) multiplet and (br) broad. Coupling constants (J) are reported in Hertz. FT-IR spectra were measured on a Perkin Elmer FT-IR spectrometer. High-resolution mass spectra were recorded on a Thermo Scientific LTQ Orbitrap-XL mass spectrometer. The purity of the compounds (>95%) was determined on a Shimadzu HPLC-PDA (column: Agilent Eclipse XDB-C8, 5 µm, 4.6 × 150 mm; λ = 254 nm, method for compounds 3 and 5–7: H₂O/CH₃CN 95:5 to 5:95 (0.1% TFA), 23 min, flow rate of 0.5 mL min⁻¹; method for compound 4: isocratic H₂O/CH₃CN 80:20 (0.1% TFA), 30 min, flow rate of 0.35 mL min⁻¹).

General procedure for the formation of the amino-pyrimidine ring in compounds 3–7. To a solution of enamine

8 (1 eq., synthesized following a previously reported procedure)¹³ in anhydrous MeOH or EtOH, the corresponding amidine (co-evaporated previously with toluene 3 times, 2–4 eq.) and NaOMe in anhydrous MeOH (2.0 M, 4–8 eq.) or NaOEt in anhydrous EtOH (2.0 M, 5 eq.) were added. The reaction mixture was stirred at reflux (95 °C, pre-heated oil bath, pressure tube) for 1–5 days. Then, the reaction mixture was cooled down, and the solvent evaporated under reduced pressure. The crude was purified by flash column chromatography.

2-(4-((4-Aminopyrimidin-5-yl)methyl)-3-methylthiophen-2-yl)ethanol (3). This compound was synthesized using enamine **8** (27.0 mg, 0.0905 mmol), anhydrous MeOH (0.22 mL), formamidine-HCl (14.5 mg, 0.181 mmol) and NaOMe (2.0 M in MeOH, 0.181 mL, 0.362 mmol). After 2 days, the same amounts of formamidine-HCl and NaOMe were added, and the reaction mixture was stirred at reflux for another 3 days (total reaction time: 5 days). The crude was purified by flash column chromatography (CH₂Cl₂/MeOH 95:5) to afford **3** as a white solid (10.0 mg, 0.0401 mmol, 46%). M. p. 155–157 °C. ¹H NMR (400 MHz, CD₃OD) δ 8.27 (s, 1H), 7.66 (s, 1H), 6.79 (s, 1H), 3.71 (t, *J* = 7.0, 2H), 3.66 (s, 2H), 2.96 (t, *J* = 7.0, 2H), 2.04 (s, 3H). ¹³C NMR (100 MHz, CD₃OD) δ 163.6 (C), 157.0 (CH), 154.1 (CH), 138.1 (C), 136.9 (C), 134.0 (C), 120.2 (CH), 117.4 (C), 63.6 (CH₂), 32.7 (CH₂), 29.2 (CH₂), 12.2 (CH₃). IR (cm⁻¹) 3446, 3316, 3144, 2928, 2852, 1646, 1592, 1553, 1484, 1412, 1374, 1323, 1254, 1124, 1059, 918, 779, 730. HRMS (ESI+) calculated for C₁₂H₁₆N₃OS [M + H]⁺ 250.1009, found 250.1008.

2-(4-((2,4-Diaminopyrimidin-5-yl)methyl)-3-methylthiophen-2-yl)ethanol (4). This compound was synthesized using enamine **8** (0.108 g, 0.364 mmol), anhydrous EtOH (1.0 mL), guanidine-HCl (68.9 mg, 0.729 mmol) and NaOEt (2.0 M in EtOH, 0.910 mL, 1.82 mmol). Reaction time: 1 day. The crude was purified by flash column chromatography (CH₂Cl₂/MeOH/Et₃N 89:10:1) to afford **4** as a yellow solid (25.5 mg, 0.0965 mmol, 27%). M. p. 246–247 °C. ¹H NMR (400 MHz, DMSO-*d*₆) δ 7.30 (s, 1H), 6.73 (s, 1H), 6.07 (s, 2H, NH₂), 5.65 (s, 2H, NH₂), 4.74 (t, *J* = 5.4, 1H, OH), 3.55–3.51 (m, 2H), 3.42 (s, 2H), 2.83 (t, *J* = 7.1, 2H), 1.98 (s, 3H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ 162.3 (C), 162.2 (C), 155.4 (CH), 139.5 (C), 134.9 (C), 132.5 (C), 117.8 (CH), 104.1 (C), 61.7 (CH₂), 31.7 (CH₂), 27.3 (CH₂), 11.9 (CH₃). IR (cm⁻¹) 3415, 3340, 3161, 2945, 1666, 1622, 1600, 1561, 1543, 1361, 1254, 1043, 1010, 987, 949, 870, 798, 742. HRMS (ESI+) calculated for C₁₂H₁₇N₄OS [M + H]⁺ 265.1118, found 265.1117.

2-(4-((4-Amino-2-(trifluoromethyl)pyrimidin-5-yl)methyl)-3-methylthiophen-2-yl)ethanol (5). This compound was synthesized using enamine **8** (80.0 mg, 0.268 mmol), anhydrous MeOH (0.64 mL), trifluoroacetamide (60.1 mg, 0.536 mmol) and NaOMe (2.0 M in MeOH, 0.535 mL, 1.07 mmol). Reaction time: 1 day. The crude was purified by flash column chromatography (CH₂Cl₂/MeOH 100:0 to 98:2) to afford **5** as a yellow solid (43.0 mg, 0.136 mmol, 51%). M. p. 125–127 °C. ¹H NMR (400 MHz, CD₃OD) δ 7.74 (s, 1H), 6.83 (s, 1H), 3.73–3.70 (m, 4H), 2.97 (t, *J* = 7.0, 2H), 2.04 (s, 3H). ¹³C NMR (100

MHz, CD₃OD) δ 164.3 (C), 155.5 (q, *J* = 35.5, C), 154.5 (CH), 137.6 (C), 137.1 (C), 133.9 (C), 121.1 (q, *J* = 274.7, CF₃), 120.4 (CH), 119.1 (C), 63.6 (CH₂), 32.7 (CH₂), 29.1 (CH₂), 12.2 (CH₃). ¹⁹F NMR (376 MHz, CD₃OD) δ -72.41. IR (cm⁻¹) 3337, 3206, 2928, 2880, 1639, 1595, 1477, 1326, 1256, 1198, 1136, 1042, 966, 739. HRMS (ESI+) calculated for C₁₃H₁₅F₃N₃OS [M + H]⁺ 318.0882, found 318.0883.

2-(4-((4-Amino-2-ethylpyrimidin-5-yl)methyl)-3-methylthiophen-2-yl)ethanol (6). This compound was synthesized using enamine **8** (80.0 mg, 0.268 mmol), anhydrous MeOH (0.64 mL), propionamide-HCl (58.2 mg, 0.536 mmol) and NaOMe (2.0 M in MeOH, 0.535 mL, 1.07 mmol). Reaction time: 2 days. The crude was purified by flash column chromatography (CH₂Cl₂/MeOH 100:0 to 98:2) to afford **6** as a yellow solid (34.1 mg, 0.123 mmol, 46%). M. p. 92–94 °C. ¹H NMR (400 MHz, CD₃OD) δ 7.60 (s, 1H), 6.78 (s, 1H), 3.70 (t, *J* = 7.0, 2H), 3.64 (s, 2H), 2.96 (t, *J* = 7.0, 2H), 2.65 (q, *J* = 7.6, 2H), 2.04 (s, 3H), 1.26 (t, *J* = 7.6, 3H). ¹³C NMR (100 MHz, CD₃OD) δ 170.6 (C), 163.9 (C), 154.4 (CH), 138.4 (C), 136.8 (C), 134.0 (C), 120.1 (CH), 114.3 (C), 63.7 (CH₂), 32.7 (CH₂), 32.5 (CH₂), 28.9 (CH₂), 13.3 (CH₃), 12.2 (CH₃). IR (cm⁻¹) 3364, 3340, 3171, 3110, 2980, 1661, 1600, 1565, 1471, 1424, 1350, 1237, 1175, 1047, 1004, 952, 798. HRMS (ESI+) calculated for C₁₄H₂₀N₃OS [M + H]⁺ 278.1322, found 278.1324.

2-(4-((4-Amino-2-isopropylpyrimidin-5-yl)methyl)-3-methylthiophen-2-yl)ethanol (7). This compound was synthesized using enamine **8** (100 mg, 0.335 mmol), anhydrous MeOH (0.80 mL), 2-methylpropanimidamide-HCl (82.1 mg, 0.670 mmol) and NaOMe (2.0 M in MeOH, 0.670 mL, 1.34 mmol). Reaction time: 2 days. The crude was purified by flash column chromatography (CH₂Cl₂/MeOH 100:0 to 99:1) to afford **7** as a yellow, sticky solid (60.2 mg, 0.207 mmol, 62%). ¹H NMR (400 MHz, CD₃OD) δ 7.60 (s, 1H), 6.77 (s, 1H), 3.70 (t, *J* = 7.0, 2H), 3.63 (s, 2H), 2.95 (t, *J* = 7.0, 2H), 2.89 (sept, *J* = 6.9, 1H), 2.03 (s, 3H), 1.24 (d, *J* = 6.9, 6H). ¹³C NMR (100 MHz, CD₃OD) δ 173.8 (C), 163.9 (C), 154.3 (CH), 138.5 (C), 136.8 (C), 134.0 (C), 120.1 (CH), 114.3 (C), 63.6 (CH₂), 37.9 (CH), 32.7 (CH₂), 29.0 (CH₂), 21.9 (2 CH₃), 12.2 (CH₃). IR (cm⁻¹) 3333, 3206, 2966, 2928, 2873, 1627, 1593, 1557, 1456, 1423, 1380, 1044, 977, 737. HRMS (ESI+) calculated for C₁₅H₂₂N₃OS [M + H]⁺ 292.1478, found 292.1480.

Expression and purification of ThiT

The expression and purification of wild-type, substrate-free ThiT were performed as described previously.¹³

Ligand-binding experiments

For compounds **3** and **5–7**, the binding affinity was determined using the intrinsic fluorescence titration assay described previously,¹³ with 50 nM ThiT in a final volume of 1000 μL of 50 mM KPi, pH 7.0, 150 mM KCl, 0.15% (w/v) *n*-decyl β-D-maltopyranoside (DM, Anatrace). For compound **4**, the binding affinity was determined by isothermal titration calorimetry (ITC) using a MicroCal iTC200 apparatus (GE

Healthcare) with a cell volume of 200 μL . The measurements were performed at 25 $^{\circ}\text{C}$ with 12.9 and 21.3 μM ThiT. A twenty-fold higher concentration of compound 4 was added in steps of 1 μL . The data were analyzed using the MicroCal LLC iTC200 software.

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