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Inhibitors of Plasmodial Serine Hydroxymethyltransferase (SHMT): Cocrystal Structures of Pyrazolopyrans with Potent Blood- and Liver-Stage Activities

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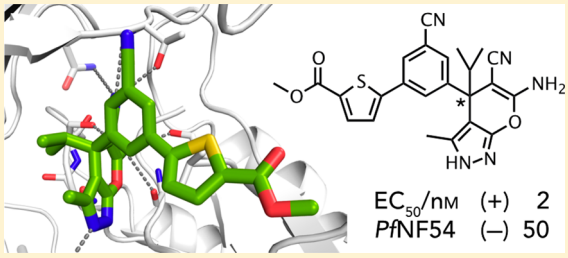
Supporting Information

ABSTRACT: Several of the enzymes related to the folate cycle are well-known for their role as clinically validated antimalarial targets. Nevertheless for serine hydroxymethyltransferase (SHMT), one of the key enzymes of this cycle, efficient inhibitors have not been described so far. On the basis of plant SHMT inhibitors from an herbicide optimization program, highly potent inhibitors of *Plasmodium falciparum* (*Pf*) and *Plasmodium vivax* (*Pv*) SHMT with a pyrazolopyran core structure were identified. Cocrystal structures of potent inhibitors with *Pv*SHMT were solved at 2.6 Å resolution. These ligands showed activity (IC_{50}/EC_{50} values) in the nanomolar range against purified *Pf*SHMT, blood-stage *Pf*, and liver-stage *P. berghei* (*Pb*) cells and a high selectivity when assayed against mammalian cell lines. Pharmacokinetic limitations are the most plausible explanation for lack of significant activity of the inhibitors in the in vivo *Pb* mouse malaria model.



INTRODUCTION

Malaria is, besides tuberculosis and AIDS, with more than 250 million clinical cases causing over 600 000 deaths per year, one of the most devastating infectious diseases worldwide.[1] By now, widespread resistance has developed against almost all available antimalarials. Even for the "last resort" artemisinin derivatives, which constitute the backbone of most new combination therapies in development, the emergence of resistance becomes a major concern.[2−8] Therefore, there is an urgent need for antimalarials with new modes of action to counteract the potentially catastrophic results of widespread artemisinin resistance, which is not unlikely based on the previous experiences with antimalarial resistance development.[9−11]

In recent years, substantial efforts have been made to identify new antimalarials using target-based approaches, which could

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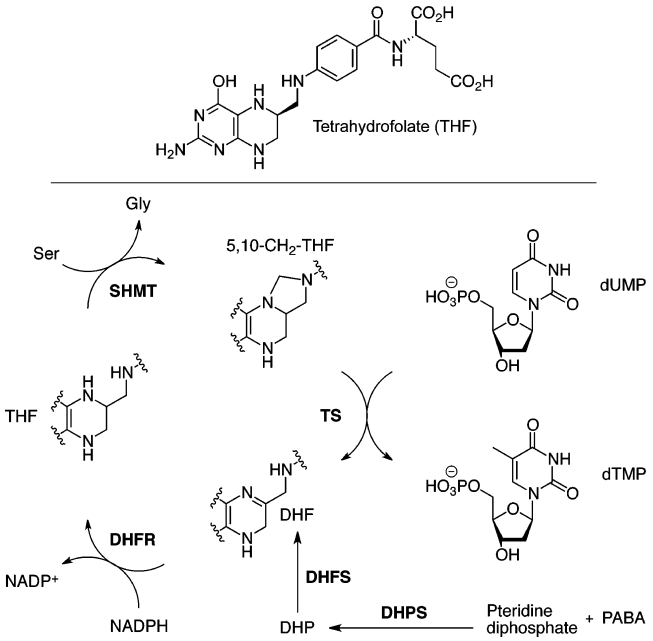
have significant advantages compared to organism-based hits due to a much better understanding of target interaction, potential resistance mechanisms, and toxicological risks. Unfortunately, most of the target-based programs, in spite of potent target activities, did not result in high activity against *P. falciparum* in cell-based assays.[12]

Plasmodium parasites have several essential enzymatic pathways in common with plants so that herbicides potentially can serve as lead structures for new antimalarials. This has been shown in a systematic screen of commercial agrochemicals against different protozoans, which resulted in a high number of promising hits.[13] Therefore, we systematically examined the antiplasmodial activity of lead structures from target-based herbicide programs at BASF.[14−16] Of these, inhibitors of the enzyme serine hydroxymethyltransferase (SHMT) raised our particular interest. SHMT plays a key role in the dTMP synthesis (Scheme 1).[17,18] As the mode of action of the antimalarial drugs

Scheme 1. Overview of the dTMP Cycle[a]



[a]Serine hydroxymethyltransferase (SHMT) converts tetrahydrofolate (THF) to 5,10-methylenetetrahydrofolate (5,10-CH_2-THF), a crucial component for the conversion of deoxyuridine monophosphate (dUMP) to the DNA precursor deoxythymidine monophosphate (dTMP) by thymidylate synthase (TS). Dihydrofolate (DHF) formed by this process is converted back to THF by dihydrofolate reductase (DHFR). In *Plasmodium*, components of the cycle are replenished by de novo synthesis: dihydropteroate synthase (DHPS) condenses pteridine diphosphate and *p*-aminobenzoic acid (*p*ABA) to dihydropteroate (DHP), which is converted to DHF by dihydrofolate synthase (DHFS).

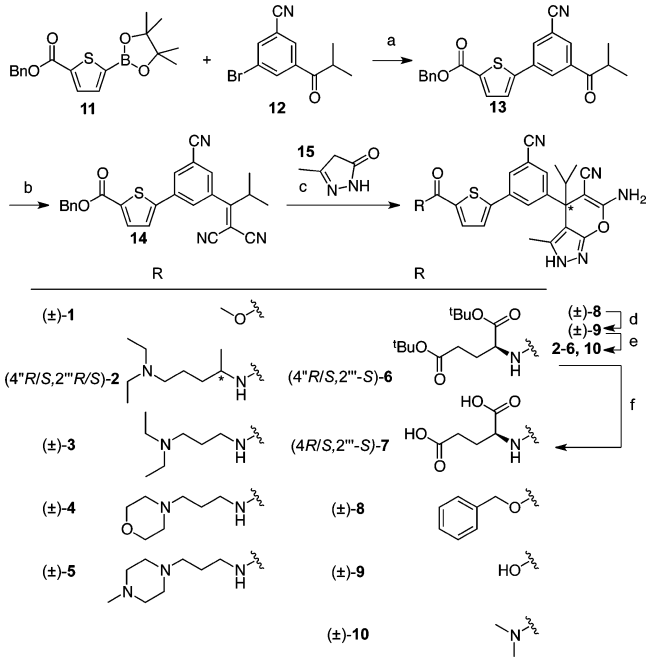
Cycloguanil, pyrimethamine, or sulfadoxine is also based on the inhibition of folate production, SHMT has been presumed to be a highly relevant target for antimalarials; however, no potent SHMT inhibitors have been described so far.[19−22]

In a screen of 100 000 compounds at BASF, two compounds with a pyrazolopyran scaffold showed IC_{50} values in the micromolar range, which could further be optimized to activities in the nanomolar range against plant SHMT.[23] These plant SHMT inhibitors were subsequently also examined as lead structures against malaria parasites.

RESULTS AND DISCUSSION

Initial Screen of BASF-Pyrazolopyran SHMT Inhibitors. A total of 338 inhibitors of plant SHMT were screened for antiplasmodial activity using a 72 h ^{3}H-hypoxanthine incorporation assay.[23] Out of these, several plant SHMT inhibitors with EC_{50} values below 100 nM in the *Pf* cell-based assay could be identified, with the most active compound (±)-**1** (Scheme 2) showing an EC_{50} value of 2.8 nM.

Scheme 2. Synthesis of Pyrazolopyran Ligands[a]



[a]Reagents and conditions: (a) $[PdCl_2(PPh_3)_2]$ (2.5 mol %), Na_2CO_3, THF/H_2O 4:1, 60 °C, 4 h, 78%; (b) malononitrile, $TiCl_4$, pyridine, $CHCl_3$, 60 °C, 4 days, 68%; (c) Et_3N, THF/MeOH 1:4, 45 °C, 6 days. (±)-**1**: 12%. (±)-**8**: 43%. (d) H_2, Pd/C, MeOH, 25 °C, 30 min, 88%; (e) CDI, then amine, THF, 25 °C, 1−3 h, 46−83%; (f) TFA, 25 °C, 14 h, quantitative. CDI = carbonyldiimidazole. TFA = trifluoroacetic acid. For atom labeling, see section S9 in the Supporting Information.

Synthesis and Cell-Based Activity of (±)-1 and Analogues. In addition to (±)-**1**, we synthesized a series of derivatives **2**−**9**, focusing in particular on the introduction of water-solubilizing and cell permeability-providing groups (Scheme 2). Ligand **2** bears a chloroquine-like substituent, whereas **7** features the side chain of tetrahydrofolate, the substrate of SHMT (for stereochemical descriptors of **2** and **7**, see Scheme 2). *N*,*N*-Dimethylamide derivative (±)-**10** addresses the hydrolysis of esters (±)-**1** and (±)-**8** under physiological conditions.

Boronic ester **11** was prepared by esterification of the corresponding thienylcarboxylate using phenyldiazomethane and reacted in a Suzuki cross-coupling with aryl bromide **12** to biaryl **13**.[24−26] A Knoevenagel condensation afforded dicyanovinyl derivative **14**, and tandem Michael addition−cyclization with an excess of pyrazolone **15** led to benzyl ester (±)-**8** featuring the pyrazolopyrane core.[27] The use of methanol as solvent, which was indispensable for the solubility of **15**, led to partial transesterification of (±)-**8** to the corresponding methyl ester (±)-**1**. Hydrogenolysis of (±)-**8** provided thienylcarboxylic acid (±)-**9**, from which the targeted amides **2**−**6**, **7** (by ester

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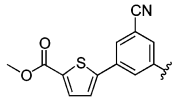
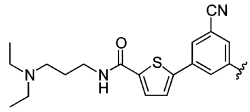
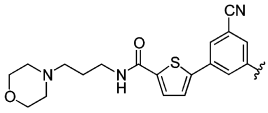
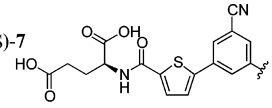
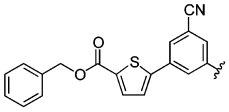
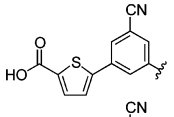
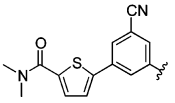
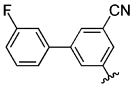
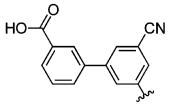
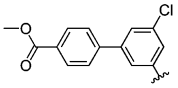
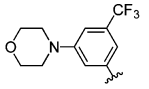
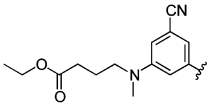
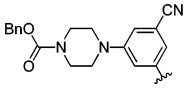
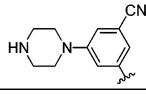
Table 1. Biological Activity of Selected Pyrazolopyran Ligands[a]

Num.	R^1	R^2	EC_{50}/nM					IC_{50}/nM
			Pf NF54	*Pf* TM90C2B	*Pf* K1	*Pf* V1/S	Rat L6	*Pf*SHMT
(±)-**1**			3	5	5	9	31100	370
(−)-**1**			49	31	35		41400	5420
(+)-**1**			2	2	3		25000	60
(±)-**3**			1160	1850	1640		96800	
(±)-**4** • 2 TFA			620	1080	1010		91400	
(4R/S,2'''S)-**7** • TFA			2520	5560	4970		>145200	
(±)-**8**			3	3	3	4	12700	420
(−)-**8**			300	240	261		11200	
(+)-**8**			2	1	1		9000	170
(±)-**9**			700	1250	1060		>224500	
(±)-**10**			59		130	92		270
(±)-**16**			1210					210
(−)-**16**			2300		2200		94000	370
(+)-**16**			2880		3630			470
(±)-**17**			340					220
(±)-**18**			80					210
(±)-**19**			600					480
(±)-**20**			920					560
(±)-**21**			2018					290
(±)-**22**			250					
(±)-**23**			4150					

[a]Data for all compounds tested are provided in the Supporting Information (Table S1).

cleavage of **6**), and **10** were obtained by condensation of the corresponding amines with carbonyldiimidazole (CDI).[28] To further improve solubility for biological testing, some of the ligands were converted into trifluoroacetate or hydrochloride salts. Detailed synthesis and characterization protocols can be found in the Supporting Information.

The cell-based activities for selected ligands are summarized in Table 1, which includes data for the newly prepared ligands and

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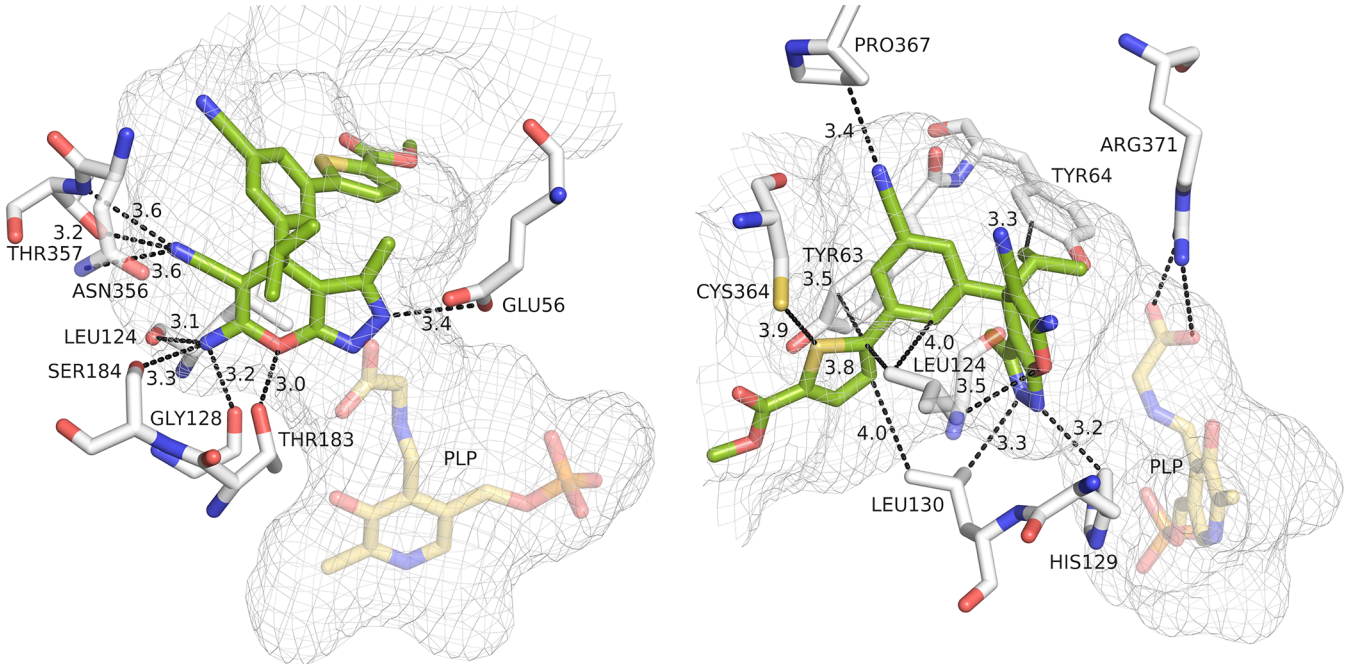

Figure 1. Cocrystal structure (PDB code 4TMR) showing protein−ligand interaction of pyrazolopyran (+)-**1** (green) and *Pv*SHMT (white). Left image shows polar (hydrogen bonding) interactions, right image nonpolar contacts. The mesh surface spans the volume of the binding pocket, and cofactor PLP is shown in yellow. Distances are given in Å. Atom coloring is the following: N blue, O red, P orange, S yellow.

other derivatives from the herbicide testing program (compounds (±)-**16** to (±)-**21**). The complete biological characterization for all compounds in this study is provided in the Supporting Information (Table S1). Methyl (±)-**1** and benzyl ester (±)-**8** show by far the most potent activity within the low single-digit nanomolar range. Replacing the thienyl against a phenyl ring (in (±)-**18**) reduces the activity to 80 nM. The *N*,*N*-dimethylamide (±)-**10** is 20-fold less active than (±)-**1**. Carboxylic acid (±)-**9** is only active in the upper nanomolar range, similar to ligand (±)-**4** with a solubilizing side chain. In general, the thienyl ring is critical for high affinity and far superior to phenyl rings, which can be rationalized on the basis of the structural data shown above.

The enantiomers of (±)-**1** and (±)-**8** were separated (enantiomeric purity >99%) by preparative HPLC on a chiral stationary phase (Daicel Chiralpak-IA 250 mm × 20 mm) using $CHCl_3$/EtOAc (95:5, 10 mL/min) as eluent. A high degree of chiral recognition was observed. The (+)-enantiomers showed a higher activity compared to their racemic mixture, whereas the activity of the (−)-enantiomers is markedly reduced (25- and 150-fold, respectively, Table 1).

***Pf* SHMT and *Pv*SHMT Enzymatic Assays.** Reaction assays of *Pf*SHMT and *Pv*SHMT were carried out using a diode array spectrophotometer according to the protocol previously reported.[19] More details are described in the Experimental Section. Table 1 shows that the inhibition of *Pf*SHMT (IC_{50} values) correlates well with the measured whole cell in vitro activities (EC_{50} values). In general, the intrinsic inhibition on the enzyme level was slightly lower (Table 1, Supporting Information Table S1 and Supporting Information section S10). Many of the compounds showed solubility problems at the highest tested concentrations, which could explain some of the differences due to the different assay characteristics. Compound (+)-**1** was also tested on *Pv*SHMT, showing an IC_{50} value of 98 ± 2 nM.

Cytotoxicity Tests. The compounds showed an excellent selectivity against mammalian L6-cells in a 72 h cytotoxicity assay with selectivity indexes for the purified enantiomers (+)-**1** and (+)-**8** of >12 000 and >4000, respectively (Table 1). In addition, cytotoxicity was tested for compound (±)-**1** in the human HepG2 cell line (human hepatocellular liver carcinoma cell line). No effects were observed up to the highest concentration (20 000 nM) tested. Distinctive structural features and kinetic properties of plasmodial and human SHMT support the observed selectivity.[29,30]

Test on Resistant *Pf* Strains. The most active hits were also tested against the resistant *P. falciparum* strains TM90C2B (resistant against chloroquine, pyrimethamine, mefloquine, atovaquone), K1 (resistant against chloroquine, sulfadoxine, pyrimethamine, cycloguanil), and V1/S (resistant against the antifolates pyrimethamine, sulfadoxine, dapsone, cycloguanil, and chloroquine) in the cell-based assay, showing almost identical activities to the sensitive strain. V1/S carries four DHFR mutations and is highly resistant to anti-DHFR drugs (Supporting Information, Table S1). Therefore, there are likely no general cross resistance issues with this compound class.

Test on *P. berghei* Liver Stage. The most active pyrazolopyran (±)-**1** has also been tested against *P. berghei* in the liver-stage assay. In brief, *Anopheles stephensi* mosquitoes infected with a transgenic luciferase-expressing *P. berghei* were obtained from the New York University Langone Medical Center Insectary (New York). A transgenic CD81-expressing HepG2 cell line (donated by Dominique Mazier) was seeded into assay plates. After ∼24 h incubation, compounds were transferred with atovaquone and puromycin used as positive controls for parasite inhibition and liver cell cytotoxicity, respectively. After 3−4 h, sporozoites (from a transgenic luciferase-expressing *P. berghei*) were added and plates were incubated at 37 °C, and following a 48 h incubation, relative luminescence was measured. Wells treated with either DMSO or positive controls were used to normalize these data for dose−

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response curve fit analysis. In this assay, (±)-**1** also showed a high potency with an IC_{50} value of 9 nM.

Animal Model and Pharmacokinetics. In initial animal models with *P. berghei* infected mice, no significant activity of (±)-**1** or (±)-**8** could be observed. Therefore, pharmacokinetic studies with (±)-**8** were conducted. After oral and intraperitoneal dosing of (±)-**8** with 100 and 10 mg/kg, respectively, plasma levels were below the limit of detection at all tested time points (1, 4, 24 h after treatment). There was evidence of rapid conversion of the ester group into the acid in the plasma samples (Supporting Information, Table S9). This potential lability of the ester group may lead into the formation of metabolite (±)-**9** with more than 200-fold reduced activity and thus could explain the lack of in vivo activity.

In liver microsomes, a rapid degradation of ester (±)-**8** and *N*,*N*-dimethylamide (±)-**10** by ester/amide hydrolysis or monooxygenation was observed (predicted hepatic extraction ratio (E_H) of 0.84 and 0.94). The closely related biphenyl analogues (±)-**17** and (±)-**38** showed improved stability (predicted E_H of 0.33 and 0.51, respectively, Supporting Information Tables S7−S10). Caco-2 studies indicate moderate permeability of (±)-**10** in the apical to basolateral direction. An efflux ratio of 5.5 suggests that (±)-**10** might be subject to efflux in the Caco-2 system which could potentially limit oral absorption (Supporting Information section S4, Table S5).

Despite its rapid metabolism, (±)-**10** showed a reduction of parasitemia of 38% (po) and 41% (ip) in a 4-day-test SCID mice model with *P. falciparum* 3D7 infected SCID mice at 4 × 30 mg/kg. Considering the very short half-life of the compound, the significant reduction of parasitemia validates the activity of pyrazolopyrans in vivo. This compound class displays folate inhibitor-like characteristics in the parasite reduction ratio assay. The profile is comparable with the DHFR inhibitor pyrimethamine (Supporting Information section S6). This result is in accordance with the observed activity decrease when reducing the duration of drug exposure in vitro. The EC_{50} values of (±)-**10** against *Pf*NF54 decrease with prolonged exposure (EC_{50} of 211, 67, and 59 nM at 24, 48, and 72 h, respectively) are similar to what can be seen with pyrimethamine (EC_{50} of 25, 7.1, and 4.6 nM at 24, 48, and 72 h, respectively). The likely stage specificity of the pyrazolopyrans is currently under investigation.

Crystal Structure Determination of *Pv*SHMT with (+)-1 and (+)-16. *Pv*SHMT was crystallized from the ternary complex of *Pv*SHMT, glycine, and either (+)-**1** or (+)-**16** (Table 1) using the microbatch method. The cocrystals diffracted to 2.7 and 2.2 Å resolution, respectively, and belong to the *C*2 space group. The structure was solved by molecular replacement using the coordinates of a chain A protomer of *Pv*SHMT (PDB code 4OYT) as the template.[31]

Binding Mode of (+)-1. Ligand (+)-**1** occupies the tetrahydrofolate (THF) binding site as seen in the crystal structure (Figure 1, PDB code 4TMR). On the basis of the structure of the complexed ligand, enantiomer (+)-**1** can be assigned the (*S*)-absolute configuration with confidence. The bicyclic pyrazolopyran core undergoes similar interactions to the aminopyrimidinone moiety of THF (Supporting Information Figure S2). A polar environment encloses the vinylogous cyanamide of (+)-**1**. The side chain and backbone of Thr357 form hydrogen bonds to the cyano group, which also interacts with Asn356. A tripod-shaped interaction is formed at the nonbasic NH_2 group with two hydrogen bonds donated to the backbone carbonyls of Leu124 and Gly128 and one accepted along the direction of the nitrogen lone pair from the side chain of Ser184. Threonine 183 forms a strong hydrogen bond to the pyran oxygen which is flanked by Leu124 on the opposing side. The N(2) of the pyrazole moiety undergoes hydrogen bonding to Glu56 and is further stabilized by van der Waals interactions with Leu130 and His129.

The plane of the bicyclic scaffold is inclined toward the *p*-aminobenzoic acid (*p*ABA) channel (Supporting Information Figure S1) opening up a small lipophilic pocket that is best filled by an isopropyl group at the stereogenic center of the core. The two methyl groups of the isopropyl residue form well aligned interactions, one pointing at the edge of Tyr64 at close distance and the other at the face of the hydrogen bonding array between Arg371 and the cofactor pyridoxal 5′-phosphate (PLP). In general, substitution of the isopropyl group by the cyclobutyl group leads to a decrease in activity (Supporting Information Table S1).

The side chain of ligand (+)-**1** extends orthogonally from the stereogenic center of the bicyclic core into the *p*ABA channel. A 1,3,5-trisubstituted phenyl group constitutes the first segment of the chain in all ligands tested. At least one of the substituents consists of a small, electron-withdrawing group (CN, Cl, CF_3). In (+)-**1**, a cyano group points into a small lateral pocket making contact with Pro367. Leucine 124 flanks the unsubstituted position para to the acceptor substituent. The second substituent of the phenyl ring further extends along the *p*ABA channel (lined by hydrophobic residues) toward the periphery of the protein. The binding mode rationalizes the experimental finding that aromatic or hydrophobic groups (Br, CF_3) can engage in effective nonpolar interactions along the channel. The 2,5-substituted thiophene moiety of (+)-**1** undergoes (in addition to CH−π interaction with Leu124 and parallel-shifted stacking with Tyr63) a sulfur−sulfur interaction with Cys364.

At the periphery, a polar aprotic headgroup provides favorable interaction with the more hydrophilic environment. The activity of esters is found far superior to that of the corresponding acids or secondary amides (Table 1, (±)-**8**, (±)-**9**, (±)-**3**).

A comparison to *Pf*SHMT apo structures (PDB code 4O6Z) shows that the CN group of the vinylogous cyanamide and N(2) of the pyrazole moiety of (+)-**1** replace two water molecules present in the ligand-free structure (Supporting Information Figure S3).[29] A third water molecule is displaced from a largely apolar environment by the isopropyl group of (+)-**1**. Favorable water displacement from a hydrophobic environment is also expected for the nitrile at the phenyl ring of (+)-**1**.

Binding Mode of (+)-16. Overall, binding mode and conformation of (+)-**16** and (+)-**1** are very similar (Figure 2, Supporting Information Figure S4 and S5). The pyrazolopyran core adopts a nearly superimposable position in the two complexes; however, some polar interactions differ because of different conformations of amino acid side chains, such as those of Thr183 and Ser184. The biphenyl substituent in (+)-**16** extending into the *p*ABA channel also assumes a conformation similar to the phenylthienyl residue in (+)-**1**. The F-substituent points into the same direction as the C═O of methyl ester (+)-**1**. The lipophilic pocket, which hosts the isopropyl substituent at the stereogenic center of (+)-**1**, is now filled by the cyclobutyl substituent of (+)-**16** but in a different orientation. The enzyme inhibition is less potent for (+)-**16** (IC_{50} = 470 nM) than for (+)-**1** (IC_{50} = 60 nM). One explanation could be the conformational difference of the surface loop, in which Cys364 is located. This loop (residues 357−367) is positioned closer to (+)-**1**, resulting in better van der Waals interactions with the cyano moiety on the phenyl ring and the thiophene ring of (+)-**1**

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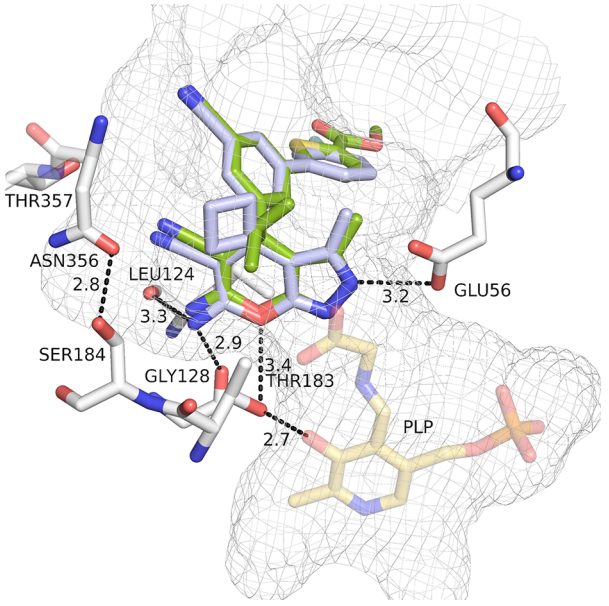

Figure 2. Cocrystal structure (PDB code 4TN4) showing polar protein−ligand interaction of pyrazolopyran (+)-**16** (blue) and *Pv*SHMT (white). Ligand (+)-**1** (green) of superimposed structure 4TMR is shown for comparison. The mesh surface spans the volume of the binding pocket, and cofactor PLP is shown in yellow. Distances are given in Å. Atom coloring is the following: F cyan, N blue, O red, P orange, S yellow.

than with the equivalent moieties of (+)-**16** (Supporting Information Figure S5). More noteworthy is the lack in chiral recognition of (±)-**16** by the enzyme. In sharp contrast to the large difference in binding affinity measured for (+)-**1** and (−)-**1**, the two enantiomers of **16** exhibit similar inhibition. In the cell-based assay, the two ligands differ strongly, with EC_{50} values of 2 nM ((+)-**1**) and 2880 nM ((+)-**16**).

Tautomerism of the Pyrazolopyran Core. The small molecule crystal structure of (±)-**10** and theoretical calculations (Supporting Information Figures S7−S10) confirm that the pyrazolopyran core is in the same tautomeric form in the free and protein-bound state. The present tautomer bears the pyrazole NH vicinal to the methyl group, enabling crucial hydrogen bonding interactions to the side chain of Glu56.

CONCLUSIONS

We have identified a novel pyrazolopyran series as potent inhibitors of plasmodial SHMT. These compounds are promising antimalarial leads, as they show high activity (IC_{50} values in the nanomolar range) on the *Pf* enzyme as well as the *Pv* enzyme and activities down to the single-digit nanomolar range in cell-based blood stage assays. The high activity in a *P. berghei* liver stage model, combined with the activity on *Pv*SHMT, makes this target especially interesting for potential treatment of blood stage malaria as well as hypnozoite stage *P. vivax* malaria.[32] The inhibitors showed a high selectivity margin relative to mammalian cell lines and no resistance against the tested multidrug resistant *Pf* strains. The main limitation so far is the low metabolic stability of the most active inhibitors. With the available cocrystal structures, a targeted optimization of the pharmacokinetic properties should now be possible, using structure-based design. Especially the variation of the exit vector including the thiophene residue appears promising, and analogs can easily be prepared in the described short synthetic sequence. Stable ester bioisosteres or alternative peripheral heteroaromatic systems should allow combination of favorable pharmacokinetic properties with high activity. Ultimately, rational scaffold variations are now possible to further extend the scope of the antimalarial SHMT inhibitors.

EXPERIMENTAL SECTION

In Vitro Antimalarial Activity. *Plasmodium falciparum* drug-sensitive NF54 and resistant K1, TM90C2B and V1/S strains were cultivated in a variation of the medium previously described, consisting of RPMI 1640 supplemented with 0.5% ALBUMAX II, 25 mM Hepes, 25 mM $NaHCO_3$ buffer (pH = 7.3), 0.36 mM hypoxanthine, and 100 μg/mL neomycin.[33,34] Human erythrocytes served as host cells. Cultures were maintained in an atmosphere of 3% O_2, 4% CO_2, and 93% N_2 in humidified modular chambers at 37 °C. Compounds were dissolved in DMSO (10 mg/mL), diluted in hypoxanthine-free culture medium, and titrated in duplicate over a 64-fold range in 96-well plates. Infected erythrocytes (1.25% final hematocrit and 0.3% final parasitemia) were added into the wells. After a 48 h incubation, 0.5 μCi [^{3}H]hypoxanthine was added, and plates were incubated for an additional 24 h. Parasites were harvested onto glass-fiber filters, and radioactivity was counted using a Betaplate liquid scintillation counter (Wallac, Zurich). The results were recorded and expressed as a percentage of the untreated controls. Fifty percent inhibitory concentrations (EC_{50}) were estimated by linear interpolation.[35]

In Vitro Cytotoxicity. Rat skeletal myoblasts (L6 cells) in RPMI 1640 medium with 10% FCS and 2 mM L-glutamine were added to each well of a 96-well microtiter plate and incubated at 37 °C under a 5% CO_2 atmosphere for 24 h. Compounds were added directly into the wells, and subsequently serial drug dilutions were prepared covering a range from 100 to 0.002 μM. The plates were incubated for another 72 h. An amount of 10 μL of Alamar Blue (12.5 mg resazurin dissolved in 100 mL water) was then added to each well, and incubation continued for a further 1−4 h. The plates were read with a Spectramax Gemini XS microplate fluorometer (Molecular Devices Cooperation, Sunnyvale, CA, USA) using an excitation wavelength of 536 nm and an emission wavelength of 588 nm. Data were analyzed using the software Softmax Pro (Molecular Devices Cooperation, Sunnyvale, CA, USA). Decrease of fluorescence (=inhibition) was expressed as percentage of the fluorescence of control cultures and plotted against the drug concentrations. From the sigmoidal inhibition curves, the IC_{50} values were calculated. Podophyllotoxin was used as positive control in the assay (IC_{50} = 0.0082 μM).

Luciferase-Expressing *Plasmodium berghei* (*Pb*-luc) Liver Stage Assay. The previously described *Plasmodium yoelii* sporozoite invasion assay (PMID, 22096101) was modified and miniaturized into a 1536-well luminescence-based assay. In brief, *Anopheles stephensi* mosquitoes infected with a transgenic luciferase-expressing *P. berghei* were obtained from the New York University Langone Medical Center Insectary (New York). A transgenic CD81-expressing HepG2 cells (gift from Dominique Mazier) was seeded at 3000 cells per well (5 μL volume in complete culturing media) into a white, 1536-well assay plate (Greiner Bio One) using a MicroFlo Microplate Dispenser (1 μL cassette; BioTek). After ∼24 h incubation at 37 °C, compounds (10 nL) were transferred using an ECHO liquid handler (Labcyte). Atovaquone and puromycin were used as positive controls for parasite inhibition and liver cell cytotoxicity, respectively. After 3−4 h, parasites obtained from freshly dissected mosquitoes were dispensed (750 parasites per well; 5 μL volume) by a BottleValve liquid dispenser (GNF Systems) equipped with a custom single-tip dispense head. The assay plates were centrifuged for 2 min at 150*g*, and then the plates were incubated at 37 °C. Following a 48 h incubation, the assay plates were inverted into collection boxes and centrifuged to remove the assay media. This was followed by a 2 μL dispense of Bright-Glo (Promega), and relative luminescence was immediately measured on an Envision (PerkinElmer). CellTiter-Glo (Promega) was added to replicate assay plates lacking parasites to determine cell viability on the Envision. Wells treated with either DMSO or positive controls were used to normalize these data for dose−response curve fit analysis.

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Enzymatic SHMT Assays. Assay mixtures (1 mL total volume) contained SHMT (~0.5 μM or 27.5 μg), L-serine (2 mM), (6S)-THF (0.4 mM), β-$NADP^+$ (0.25 mM), and the coupling enzyme methylene tetrahydrofolate dehydrogenase (FolD, 5 μM or 155 μg) in 50 mM HEPES, pH 7.0, containing 1 mM DTT and 0.5 mM EDTA. To mixtures was added 10 μL of inhibitors with various concentrations (final concentrations from 1 to 1000 nM), and initial rates of the reaction were monitored to measure the amount of noninhibited enzyme. The inhibitors were dissolved in DMSO, and the control assays without inhibitor but in the presence of 1% DMSO (final concentration) were also carried out.

Crystallization of Recombinant *Pv*SHMT and Compounds (+)-1 and (±)-16. *Pv*SHMT was crystallized using microbatch method in a 60-well plate (⌀ 1 mm at bottom of each well) covered with 6 mL of baby oil (Johnson; a mixture of mineral oil, olive oil, and vitamin E, PZ Johnson, Thailand). Protein−ligand complexes were prepared by mixing 60 μL of purified *Pv*SHMT protein (20−25 mg/mL) with 1 mM PLP, 60 mM β-mercaptoethanol, 90 mM glycine, and 3.2 mM (+)-**1** or (±)-**16**. The mixture was equilibrated on ice for 30 min to allow for complete complex formation. The crystallization drop is composed of 1 μL each of a crystallization solution and the protein complex. Protein crystals of *Pv*SHMT were grown at 293 K in 20−24% w/v PEG4000, 0.06−0.12 M NaCl, 0.1 M Tris-HCl buffer, pH 8.5, and additive 10% v/v trifluoroethanol.

***Pv*SHMT Crystal Structure Data Collection, Structure Determination, and Refinement.** A single crystal was flash-vitrified in liquid nitrogen using 20% glycerol in crystallizing agent as a cryoprotectant. X-ray diffraction data were collected at 100 K at wavelength of 1 Å using ADSC Quantum-315 CCD detector at beamline 13B1, NSRRC, Taiwan. Data were processed using HKL2000 package. X-ray diffraction data and refinement statistics are listed in Supporting Information Table S12. The structure of *Pv*SHMT was determined by molecular replacement using Phaser in CCP4 suite with a chain A protomer of *Pv*SHMT coordinate (PDB code 4OYT) as the template. Model building and structure refinement were carried out using Coot and Refmac5. The ligand structure was prepared using HYPERCHEM.

Animal Models. All in vivo studies conducted at the SwissTPH were adhering to local and national regulations of laboratory animal welfare in Switzerland (permission numbers 1731 and 2303). *P. berghei* in vivo antimalarial activity was assessed for groups of five female NMRI mice (20−22 g) intravenously infected on day 0 with 2×10^7 erythrocytes parasitized with GFP parasites (PbGFPCON, kindly donated by Drs. A. P. Waters and C. J. Janse, University of Glasgow and Leiden University).[36] From historical data, untreated control mice died typically between day 6 and day 7 postinfection, but in these studies all animals showing unabated parasitemia or malaria symptoms were euthanized on day 4. The experimental compound was formulated in 7% v/v Tween 80/3% v/v ethanol. Compounds were administered intraperitoneally or orally in a 200 μL volume of 10 mL/kg as a single dose (24 h postinfection) or as four consecutive doses (6, 24, 48, and 72 h postinfection). We determined parasitemia at 96 h postinfection, using standard flow cytometry techniques. Activity was calculated as the difference between the mean percent parasitemia for the control and treated groups expressed as a percentage of the control group. The survival time in days was also recorded up to 30 days after infection. A compound was considered curative if the animal survived to day 30 after infection with no detectable parasites.

To exclude differences between both *Plasmodium* species, the in vivo antimalarial activity was also studied in the SCID mouse *P. falciparum* model. This recently established model uses SCID mice engrafted with human erythrocytes, offering the possibility to investigate *P. falciparum* in vivo.[37,38]

In Vivo Pharmacokinetics Description. For in vivo PK studies, female NMRI mice (20−25 g) were obtained from Harlan Laboratories (The Netherlands) and were randomly assigned to cages. Mice were allowed to acclimate before initiation of the experiments. Feed and water were given ad libitum. Compounds were formulated at concentrations of 10 mg/mL for a dose of 100 mg/kg administered orally (po) and at a concentration of 1 mg/mL for a dose of 10 mg/kg given intraperitoneally (ip). The solution formulation for po and ip dosing contained 3% ethanol and 70% Tween 80. Blood samples from mice were collected at 1, 4, and 24 h after dosing. Groups of four mice were used for each time point. An amount of 50 μL of blood was collected from each of the four mice, pooled, and centrifuged at 13 000 rpm for 7 min at 4 °C. Lithium heparin plasma was harvested and stored at −80 °C until analysis. For detailed analytic procedure see Supporting Information.

Chemical Synthesis: General Methods. Commercially available chemicals (Abcr, Aldrich, Acros, Fluka, Fischer, Maybridge, Sigma, TCI) were used without further purification. Analytic thin layer chromatography (TLC) was conducted on SiO_2 60 F_{254} coated glass plates (Merck Millipore) and on F_{254} Al_2O_3 coated glass plates (Merck Millipore). Fluorescence extinction was detected at 254 nm, fluorescence at 366 nm. Preparative thin layer chromatography was conducted on glass plates with a SiO_2 60 F_{254} coating of 1 mm thickness (SiliCycle). Infrared (IR) spectra were recorded on a PerkinElmer Spectrum Two FT-IR device fitted with an ATR unit (4000−600 cm^{-1}). Absorption bands are given in wavenumbers (cm^{-1}), and signal intensities are labeled with s (strong), m (medium), and w (weak). Solvents used for chromatography and extraction were distilled prior to use. Reactions were carried out in puriss. or p.a. grade solvents. ^1H and ^{13}C NMR spectra were recorded on Varian Gemini 300 (300/75 MHz), Bruker AVIII 300 (300/75 MHz), Bruker DRX 400 (400/100 MHz), or Bruker DRX 600 (600/150 MHz). All spectra were measured at 300 K. Deuterated solvents were obtained from Armar Chemicals. Chemical shifts are referenced to solvent residual peaks ($(CD_3)_2CO$, δ H = 2.05, δ C = 29.84 ppm; $CDCl_3$, δ H = 7.26, δ C = 77.16 ppm; $(CD_3)_2SO$, δ H = 2.50, δ C = 39.52 ppm; CD_3CN, δ H = 1.94, δ C = 1.32 ppm; CD_3OD, δ H = 3.31, δ C = 49.00 ppm; (D_8)THF, δ H = 3.58, δ C = 67.57 ppm). The spin multiplicity is abbreviated as s (singlet), d (doublet), t (triplet), combinations of such, or m (multiplet). Coupling constants are denoted by *J* and are given in Hz. Broad signals are annotated with br. ^{13}C spectra are broadband decoupled. Assignments of ^1H and ^{13}C signals are supported by 2D NMR experiments (COSY, HSQC, HMBC, INADEQUATE). The nomenclature was obtained with the computer program ACD/Name (ACD/Labs). Medium pressure liquid chromatograpy (MPLC) was carried out on a Teledyne Isco CombiFlash system using Teledyne RediSep SiO_2 cartridges or on a Büchi Sepacore system using self-packed columns (C_{18} end-capped reversed phase silica gel, Fluka 60756). Melting points were measured on a Büchi M560 device. High-resolution electrospray ionization mass spectrometry (HR-ESI-MS) was conducted with a Bruker maXis ESI/Nanospray Qq TOF instrument. All mass spectra were acquired by the MS-Service of the Laboratory of Organic Chemistry (ETH Zurich). Liquid chromatography/mass spectrometry (LC/MS) was performed on a Dionex UltiMate 3000 LC instrument equipped with a Dionex MSQ Plus mass spectrometer. Agilent ZORBAX Eclipse Plus C18 columns (30 mm × 3 mm; 3.5 μm pore size) were used.

Chemical Synthesis: Purification Procedure 1 (PP1). Purification of Amines by Acid−Base Extraction. Extraction was carried out in two centrifuge tubes (15 mL) for quick phase separation through centrifugation. The crude product was suspended in EtOAc (10 mL) under sonication and washed twice with aqueous NH_3 solution (10 mL, pH 11). The organic phase was extracted with aqueous HCl solution (10 mL, pH 2). The organic phase was discarded. The acidic aqueous phase was adjusted to pH 11 with aqueous NH_3 solution (leading to precipitation) and extracted twice with EtOAc (10 mL). The basic aqueous phase was discarded. The organic phase was concentrated, and the residue was dried under high vacuum. The obtained product may still contain traces of imidazole which can be removed via PP2.

Chemical Synthesis: Purification Procedure 2 (PP2). Purification of Amides by RP-MPLC. To remove traces of imidazole, reversed phase chromatography was carried out on a 12 mm × 55 mm column packed with C_{18} end-capped reversed phase silica gel (Fluka 60756). Eluent mixtures composed of variable amounts of H_2O (0.01% NH_3, pH 9.5) and MeCN at a flow rate of 30 mL/min were used. Storing columns under basic conditions leads to hydrolysis of the C_{18} modification and should therefore be avoided.

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Chemical Synthesis: General Procedure 1 (GP1). CDI Mediated Synthesis of Amides.[28] A solution of acid (±)-**9** (1.0 equiv) in dry THF (54 mg/mL) was treated with a suspension of carbonyldiimidazole (CDI) (2.0 equiv) in dry THF (50 mg/mL) and stirred for 30 min at 25 °C. Completion of the reaction was verified via LC−MS by concentrating a small sample and dissolving the residue in neat MeCN. The suspension was filtered through a syringe filter to remove excess CDI. The solution was treated with a variable amount of a solution of amine compound in THF and was stirred for a variable time at a variable temperature. Completion of the reaction was verified via LC−MS. The solution was concentrated and the residue subjected to PP1 and PP2 for purification.

Chemical Synthesis: General Procedure 2 (GP2). TFA Salts of Amines. To increase the solubility and facilitate handling, insoluble compounds containing amines were converted to the trifluoroacetates. An excess of TFA was added to the solution/suspension/solid, followed by distilling off excess TFA under vacuum. Drying under high vacuum offers the pure desired salt.

Chemical Synthesis: Characterization and Spectra. Reference data for compounds commercially available or synthesized according to literature procedures are provided in Supporting Information section S1.1. Full characterization of compounds reported in patent application WO 2013182472 A1 ((±)-**16** to (±)-**21** and (±)-**35** to (±)-**48**) is provided in the Supporting Information section S1.2. For NMR spectra, structure, and atom numbering of all reported compounds see Supporting Information section S9.

(±)-Benzyl 5-[3-(6-Amino-5-cyano-3-methyl-4-isopropyl-2,4-dihydropyrano[2,3-*c*]pyrazol-4-yl)-5-cyanophenyl]thiophene-2-carboxylate ((±)-8) and (±)-Methyl 5-[3-(6-Amino-5-cyano-4-isopropyl-3-methyl-1,4-dihydropyrano[2,3-*c*]pyrazol-4-yl)-5-cyanophenyl]thiophene-2-carboxylate ((±)-1, CAS 1508291-70-0). A solution of **14** (1.94 g, 4.43 mmol) in dry THF (12 mL) was added to a solution of 3-methyl-5-pyrazolone **15** (2.17 g, 22.2 mmol) in dry MeOH (50 mL). Et_3N (0.22 g, 2.17 mmol) was added resulting in an orange solution. The mixture was degassed, stirred under argon for 6 days at 45 °C, treated with SiO_2 (10 g), and concentrated. Chromatography on a Teledyne CombiFlash MPLC system (RediSep 80 g SiO_2, dry loading, gradient EtOAc/cyclohexane 20:80 to 50:50 over 30 min) gave (±)-**8** (1.02 g, 43%) and (±)-**1** (0.25 g, 12%) as yellow foams. Data of (±)-**8**: R_f = 0.36 (SiO_2, EtOAc/CH_2Cl_2 40:60); mp 133−139 °C. ^{1}H NMR (600 MHz, (D_8)THF): δ = 0.88 and 0.98 (2 d, J = 6.6 Hz, 6 H; CHMe_2), 1.85 (s, 3 H; Me−C(3″)), 2.89 (heptet, J = 6.6 Hz, 1 H; CHMe$_2$), 5.33 (s, 2 H; CH_2Ph), 6.30 (br s, 2 H; NH_2), 7.28−7.32 (br t, J = 7.3 Hz, 1 H; H−C(4) of Ph), 7.33−7.37 (br t, J = 7.3 Hz, 2 H; H−C(3, 5) of Ph), 7.43−7.45 (br d, J = 7.3 Hz, 2 H; H−C(2, 6) of Ph), 7.54 (d, J = 3.9 Hz, 1 H; H−C(4)), 7.72 (br t, J = 1.6 Hz, 1 H; H−C(4′)), 7.79 (d, J = 3.9 Hz, 1 H; H−C(3)), 7.95 (br t, J = 1.8 Hz, 1 H; H−C(2′)), 8.00 (br t, J = 1.6 Hz, 1 H; H−C(6′)), 11.35 ppm (br s, 1 H; NH). ^{13}C NMR (150 MHz, (D_8)THF, INADEQUATE): δ = 12.30 (^1J(C,C) = 51.5 Hz; Me−C(3″)), 18.90 (^1J(C,C) = 36.4 Hz) and 19.41 (^1J(C,C) = 36.7 Hz; CHMe_2), 36.25 (^1J(C,C) = 34.4, 32.7 Hz; CHMe$_2$), 48.86 (^1J(C,C) = 47.7, 43.9, 32.5 Hz; C(4″)), 62.60 (^1J(C,C) = 87.9 Hz; C(5″)), 67.58 (^1J(C,C) = 48.8 Hz; CH_2Ph), 99.65 (^1J(C,C) = 72.4, 44.2 Hz; C(3″a)), 114.5 (^1J(C,C) = 80.2, 59.0, 58.3 Hz; C(5′)), 119.01 (^1J(C,C) = 80.1 Hz; NC−C(5′)), 120.29 (NC−C(5″)), 126.47 (^1J(C,C) = 62.5, 58.3 Hz; C(4)), 128.56 (^1J(C,C) = 64.5, 58.0 Hz; C(6′)), 129.11 (C(4) of Ph), 129.18 (^1J(C,C) = 57.6 Hz; C(2, 6) of Ph), 129.42 (C(3, 5) of Ph), 131.02 (^1J(C,C) = 64.2, 58.4 Hz; C(2′)), 133.13 (^1J(C,C) = 59.2, 58.1 Hz; C(4′)), 134.58 (^1J(C,C) = 87.6, 64.2 Hz; C(2)), 135.39 (^1J(C,C) = 65.7, 58.9, 58.7 Hz; C(1′)), 135.50 (^1J(C,C) = 64.8, 52.5 Hz; C(3)), 136.13 (^1J(C,C) = 73.8, 50.3 Hz; C(3″)), 137.29 (^1J(C,C) = 56.9, 49.5 Hz; C(1) of Ph), 148.40 (^1J(C,C) = 64.1, 64.1, 42.9 Hz; C(3′)), 149.63 (^1J(C,C) = 63.9, 61.8 Hz; C(5)), 157.43 (C(7″a)), 162.07 (^1J(C,C) = 87.7 Hz; CO_2), 163.05 ppm (^1J(C,C) = 94.7 Hz; C(6″)). IR (ATR): ν = 3319 (w), 3194 (w), 3093 (w), 2969 (w), 2879 (w), 2232 (w), 2186 (m), 1704 (m), 1632 (s), 1585 (s), 1535 (m), 1487 (m), 1465 (m), 1420 (m), 1383 (s), 1344 (m), 1281 (s), 1243 (s), 1175 (m), 1153 (m), 1093 (s), 1042 (s), 1001 (m), 940 (m), 907 (m), 865 (m), 820 (m), 783 (w), 745 cm^{-1} (s). HR-ESI-MS m/z (%): 537.1782 (42), 536.1750 (100, [M + H]$^+$; calcd for $C_{30}H_{26}N_5O_3S^+$, 536.1751). Data and alternative synthesis of (±)-**1** are given in a separate procedure. Separation of (±)-**8** and (±)-**1** enantiomers by chiral phase HPLC (column, Daicel, Chiralpak-IA 250 mm × 20 mm; flow, 10 mL/min; detection, 254 nm; eluent, $CHCl_3$/EtOAc (95:5); sample dissolved in eluent (∼6 mg/mL) and injected. (−)-**8**: t_R = 5.80 min, $[\alpha]^{28}_D$ −117° (c 0.058, $CHCl_3$). (+)-**8**: t_R = 6.48 min, $[\alpha]^{28}_D$ 128° (c 0.065, $CHCl_3$). (−)-**1**: t_R = 6.20 min, $[\alpha]^{28}_D$ −117° (c 0.067, $CHCl_3$). (+)-**1**: t_R = 7.18 min, $[\alpha]^{28}_D$ 128° (c 0.069, $CHCl_3$).

(±)-Methyl 5-[3-(6-Amino-5-cyano-4-isopropyl-3-methyl-1,4-dihydropyrano[2,3-*c*]pyrazol-4-yl)-5-cyanophenyl]-thiophene-2-carboxylate ((±)-1, CAS 1508291-70-0). A mixture of **32** (405 mg, 1.1 mmol), 3-methyl-1*H*-pyrazol-5(4*H*)-one **15** (110 mg, 1.1 mmol), and 2 drops of Hünig's base in MeOH/THF 1:1 (6 mL) was stirred for 48 h at 50 °C and evaporated. The residue was taken up in EtOAc (50 mL) and washed with water (3 × 50 mL). The organic phase was dried over Na_2SO_4 and evaporated. Column chromatography (SiO_2; n-hexane/EtOAc 4:1 → 1:1) and subsequent crystallization gave (±)-**1** (40 mg, 8%) as an off-white solid. R_f = 0.11 (SiO_2; n-hexane/EtOAc 1:1); mp 186−187 °C. ^{1}H NMR (400 MHz, CD_3OD): δ = 0.90 and 1.02 (2 d, J = 6.5 Hz, 6 H; CHMe_2), 1.86 (s, 3 H; Me−C(3″)), 2.89 (heptet, J = 6.6 Hz, 1 H; CHMe$_2$), 3.89 (s, 3 H; OMe), 7.52 (br d, J = 4.0 Hz, 1 H; H−C(4)), 7.76 (br t, J = 1.5 Hz, 1 H; H−C(4′)), 7.78 (br d, J = 4.0 Hz, 1 H; H−C(3)), 7.92 (br t, J = 1.7 Hz, 1 H; H−C(2′)), 7.98 ppm (br t, 1 H; H−C(6′)). ^{13}C NMR (100 MHz, CD_3OD): δ = 11.93 (Me−C(3″)), 18.72 and 19.22 (CHMe_2), 36.49 (CHMe$_2$), 52.84 (OMe), 61.70 (C(5″)), 100.31 (C(3″a)), 114.56 (C(5′)), 119.19 (NC−(5′)), 121.58 (NC−(5″)), 126.77 (C(4)), 128.85 (C(6′)), 131.73 (C(2′)), 133.14 (C(4′)), 134.66 (C(2)), 135.80 (C(3)), 135.91 (C(1′)), 137.52 (C(3″)), 148.47 (C(3′)), 149.56 (C(5)), 157.50 (C(7″a)), 163.69 (CO_2), 164.08 (C(6″)) ppm, C(4″) obscured by solvent signal of CD_3OD. IR (ATR): $\tilde{\nu}$ = 3078 (w), 2120 (w), 1714 (m), 1636 (m), 1464 (m), 1434 (m), 1366 (w), 1287 (s), 1154 (w), 1101 (s), 975 (w), 953 (w), 889 (w), 820 (m), 749 (s), 689 (m), 648 (m), 627 cm^{-1} (m). HR-MALDI-MS m/z (%): 460.1438 (100, [M + H]$^+$; calcd for $C_{24}H_{22}N_5O_3S^+$, 460.1438).

(4″*R*/*S*,2‴*R*/*S*)-5-[3-(6-Amino-5-cyano-3-methyl-4-isopropyl-2,4-dihydropyrano[2,3-*c*]pyrazol-4-yl)-5-cyanophenyl]-*N*-[5-(diethylamino)pent-2-yl]thiophene-2-carboxamide (4″*R*/*S*,2‴*R*/*S*-2). Following GP1, the active ester of (±)-**9** (0.34 mmol) and 2-amino-5-diethylaminopentane (159 mg, 1 mmol) were reacted at 25 °C over 10 min. Purification according to PP1 yielded (4″*R*/*S*,2‴*R*/*S*)-**2** (166 mg, 83%) contaminated with traces of imidazole, which were separated according to PP2 (eluent H_2O/MeCN 65:35, change to 55:45 after 8 min). R_f = 0.37 (Al_2O_3; EtOH/EtOAc 10:90); mp 128−139 °C. ^{1}H NMR (400 MHz, CD_3CN): δ = 0.84 and 0.956 (2 d, J = 6.6−6.7 Hz, 6 H; CHMe_2), 0.957 (t, J = 7.1 Hz, 6 H; N(CH_2Me)$_2$), 1.19 (d, J = 6.6 Hz, 3 H; NHCHMe), 1.42−1.59 (m, 4 H; NHCHCH_2CH_2), 1.80 (s, 3 H; Me−C(3″)), 2.40 (t, J = 7.0 Hz, 2 H; NCH_2CH$_2$), 2.46 (q, J = 7.1 Hz, 4 H; N(CH_2Me)$_2$), 2.84 (heptet, J = 6.7 Hz, 1 H; CHMe$_2$), 4.05 (br heptet, J = 6.8 Hz, 1 H; NHCH), 5.46 (s, 2 H; NH_2), 6.98 (d, J = 8.4 Hz, 1 H; CONH), 7.42 (d, J = 3.9 Hz, 1 H; H−C(4)), 7.53 (d, J = 3.9 Hz, 1 H; H−C(3)), 7.75 (t, J = 1.5 Hz, 1 H; H−C(4′)), 7.88 (t, J = 1.9 Hz, 1 H; H−C(2′)), 7.93 (t, J = 1.6 Hz, 1 H; H−C(6′)), 10.15−10.65 ppm (br s, 1 H; H−N(2″)). ^{13}C NMR (100 MHz, CD_3CN): δ = 12.08 (Me−C(3″)), 12.18 (N(CH_2Me)$_2$), 18.63 and 19.07 (CHMe_2), 21.20 (NHCHMe), 24.58 (CH_2CH_2CH_2), 35.13 (NHCHCH_2), 35.99 (CHMe$_2$), 46.66 (NHCH), 47.64 (N(CH_2Me)$_2$), 48.57 (C(4″)), 53.36 (NCH_2CH$_2$), 63.48 (C(5″)), 99.99 (C(3″a)), 113.98 (C(5′)), 119.27 (NC−C(5′)), 120.64 (NC−C(5″)), 126.47 (C(4)), 128.65 (C(6′)), 129.28 (C(3)), 131.20 (C(2′)), 132.63 (C(4′)), 135.63 (C(1′)), 137.03 (C(3″)), 141.98 (C(2)), 146.21 (C(5)), 147.84 (C(3′)), 157.07 (C(7″a)), 161.53 (CONH), 162.89 ppm (C(6″)). IR (ATR): ν = 3300 (w), 3168 (w), 3090 (w), 2968 (m), 2933 (m), 2874 (w), 2818 (w), 2232 (w), 2185 (m), 1630 (s), 1585 (s), 1544 (s), 1520 (s), 1486 (s), 1386 (s), 1289 (s), 1149 (m), 1069 (m), 1043 (m), 990 (m), 884 (m), 817 (m), 734 (m), 700 cm^{-1} (m). HR-ESI-MS m/z (%): 587.2986 (38), 586.2955 (100, [M + H]$^+$; calcd for $C_{32}H_{40}N_7O_2S^+$, 586.2959).

(±)-5-[3-(6-Amino-5-cyano-3-methyl-4-isopropyl-2,4-dihydropyrano[2,3-*c*]pyrazol-4-yl)-5-cyanophenyl]-*N*-[3-

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(diethylamino)propyl]thiophene-2-carboxamide ((±)-3). Following GP1, the active ester of (±)-**9** (0.15 mmol) and 3-(diethylamino)propylamine (33 mg, 0.25 mmol) were reacted at 25 °C over 20 min. Purification according to PP1 yielded (±)-**3** (71 mg, 83%) contaminated with traces of imidazole, which were separated according to PP2 (eluent H_2O/MeCN 65:35, change to 60:40 after 6 min). A small fraction of (±)-**3** was converted to the ditrifluoroacetate (±)-**3a** according to GP2. Data of (±)-**3**: mp 127−134 °C. ^{1}H NMR (400 MHz, CD_3CN): δ = 0.85 and 0.96 (2 d, J = 6.6 Hz, 6 H; CHMe_2), 1.03 (t, J = 7.1 Hz, 6 H; N(CH_2Me)$_2$), 1.72 (quint, J = 6.3 Hz, 2 H; $CH_2CH_2CH_2$), 1.80 (s, 3 H; Me−C(3″)), 2.561 (q, J = 7.2 Hz, 4 H; N(CH_2Me)$_2$), 2.573 (t, J = 5.9 Hz, 2 H; NCH_2CH_2), 2.85 (heptet, J = 6.6 Hz, 1 H; CHMe$_2$), 3.41 (td, J = 6.3, 4.8 Hz, 2 H; NHCH_2), 5.46 (s, 2 H; NH_2), 7.43 (d, J = 3.9 Hz, 1 H; H−C(4)), 7.48 (d, J = 3.9 Hz, 1 H; H−C(3)), 7.75 (t, J = 1.6 Hz, 1 H; H−C(4′)), 7.87 (t, J = 1.8 Hz, 1 H; H−C(2′)), 7.94 (t, J = 1.6 Hz, 1 H; H−C(6′)), 8.27 (br t, J = 5.0 Hz, 1 H; CONH), 10.14−10.67 ppm (br s, 1 H; H−N(2″)). IR (ATR): ν = 3301 (w), 3171 (w), 3089 (w), 2968 (m), 2933 (m), 2874 (w), 2821 (w), 2232 (w), 2185 (m), 1632 (s), 1585 (s), 1547 (s), 1520 (s), 1486 (s), 1386 (s), 1294 (s), 1168 (m), 1044 (m), 903 (m), 883 (m), 818 (m), 734 cm^{-1} (m). HR-ESI-MS m/z (%): 559.2674 (34), 558.2643 (100, [M + H]$^+$; calcd for $C_{30}H_{36}N_7O_2S^+$: 558.2646). Data of (±)-**3a**: R_f = 0.29 (Al_2O_3; EtOH/EtOAc 10:90); mp 65−69 °C. ^{1}H NMR (400 MHz, CD_3CN): δ = 0.85 and 0.96 (2 d, J = 6.7 Hz, 6 H; CHMe_2), 1.27 (t, J = 7.3 Hz, 6 H; HN$^+$(CH_2Me)$_2$), 1.80 (s, 3 H; Me−C(3″)), 1.96−2.02 (quint, 2 H; $CH_2CH_2CH_2$), 2.85 (heptet, J = 6.7 Hz, 1 H; CHMe$_2$), 3.10 (br q, J = 6.5 Hz, 2 H; HN$^+C$$H_2CH_2$), 3.14−3.22 (m, 4 H; HN$^+$($CH_2$Me)$_2$), 3.45 (br q, J = 6.2 Hz, 2 H; CONHCH_2), 5.46 (br s, 2 H; NH_2), 7.48 (d, J = 4.0 Hz, 1 H; H−C(4)), 7.58 (br t, J = 6.8 Hz, 1 H; CONH), 7.62 (d, J = 4.0 Hz, 1 H; H−C(3)), 7.78 (br t, J = 1.6 Hz, 1 H; H−C(4′)), 7.89 (br t, J = 1.8 Hz, 1 H; H−C(2′)), 7.95 (br t, J = 1.6 Hz, 1 H; H−C(6′)), 8.10−8.35 ppm (br s, 1 H; $(CH_2)_3NH^+$). ^{13}C NMR (100 MHz, CD_3CN): δ = 9.03 (HN$^+$(CH_2Me)$_2$), 12.06 (Me−C(3″)), 18.62 and 19.05 (CHMe_2), 25.41 ($CH_2CH_2CH_2$), 36.01 (CHMe$_2$), 36.83 (NHCH_2), 48.00 (HN$^+$(CH_2Me)$_2$), 48.59 (C(4″)), 50.11 (HN$^+C$$H_2CH_2$), 63.44 (C(5″)), 100.03 (C(3″a)), 114.08 (C(5′)), 115.08 (q, ^1J(C,F) ≈ 270 Hz, CF_3), 119.20 (NC−C(5′)), 120.61 (NC−C(5″)), 126.87 (C(4)), 128.88 (C(6′)), 130.80 (C(3)), 131.30 (C(2′)), 132.97 (C(4′)), 135.30 (C(1′)), 137.14 (C(3″)), 139.54 (C(2)), 147.59 (C(5)), 147.92 (C(3′)), 157.01 (C(7″a)), 159.51(q, ^2J(C,F) = 38.9 Hz, CF_3CO_2), 162.89 (C(6″)), 164.34 ppm (CONH). IR (ATR): ν = 3320 (w), 2973 (w), 2234 (w), 2188 (w), 1776 (w), 1633 (m), 1587 (m), 1551 (m), 1487 (m), 1392 (m), 1308 (m), 1141 (s), 1036 (m), 813 (m), 703 cm^{-1} (s).

(±)-6-Amino-5-cyano-4-[3-cyano-5-(5-{[3-(morpholin-4-ium-4-yl)propyl]carbamoyl}thiophen-2-yl)phenyl]-3-methyl-4-isopropyl-2,4-dihydropyrano[2,3-*c*]pyrazol-1-ium Bis(trifluoroacetate) ((±)-4a). Following GP1, the active ester of (±)-**9** (0.15 mmol) and 3-morpholinopropylamine (37 mg, 0.25 mmol) were reacted at 25 °C over 20 min. Purification according to PP1 yielded (±)-**4** (77 mg, 87%) contaminated with traces of imidazole, which were separated according to PP2 (eluent H_2O/MeCN 70:30). Note that for PP1 the precipitate in the acidic aqueous phase is insoluble product, which dissolves again in the organic phase upon raising the pH. For better solubility (±)-**4** was converted to the ditrifluoroacetate (±)-**4a** according to GP2. Data of (±)-**4a**: R_f = 0.31 (Al_2O_3; EtOH/EtOAc 10:90); mp 71−76 °C. ^{1}H NMR (400 MHz, CD_3CN): δ = 0.85 and 0.96 (2 d, J = 6.5 Hz, 6 H; CHMe_2), 1.80 (s, 3 H; Me−C(3)), 2.02 (br quint, J ≈ 6.5 Hz, 2 H; $CH_2CH_2CH_2$), 2.84 (heptet, J = 6.6 Hz, 1 H; CHMe$_2$), 3.02 (br t, J ≈ 12.2 Hz, 2 H; H_{ax}−C(3, 5) of morpholinium), 3.15 (br t, J = 6.9 Hz 2 H; HN$^+C$$H_2CH_2CH_2$); 3.41 (br d, J = 12.9 Hz, 2 H; H_{eq}−C(3, 5) of morpholinium), 3.42−3.49 (m, 2 H; CONHCH_2), 3.79 (br td, J = 12.5, 1.9 Hz, 2 H; H_{ax}−C(2, 6) of morpholinium), 4.02 (br dd, J = 13.4, 3.0 Hz, 2 H; H_{eq}−C(2, 6) of morpholinium), 5.48 (br s, 2 H; NH_2), 7.46 (d, J = 4.0 Hz, 1 H; H−C(3″)), 7.63 (d, J = 4.0 Hz, 1 H; H−C(4″)), 7.68 (br t, J = 6.0 Hz, 1 H; CONH), 7.77 (t, J = 1.6 Hz, 1 H; H−C(2′)), 7.89 (t, J = 1.8 Hz, 1 H; H−C(6′)), 7.94 (t, J = 1.5 Hz, 1 H; H−C(4′)), 9.82 ppm (s, 1 H; $(CH_2)_3NH$+). ^{13}C NMR (100 MHz, CD_3CN): δ = 12.07 (Me−C(3)), 18.63 and 19.05 (CHMe_2), 24.59 ($CH_2CH_2CH_2$), 36.01 (CHMe$_2$), 36.94 (CONHCH_2), 48.58 (C(4)), 52.78 (C(3, 5) of morpholinium), 55.23 (HN$^+C$$H_2CH_2$), 63.41 (C(5)), 64.79 (C(2, 6) of morpholinium), 100.01 (C(3a)), 114.05 (C(3′)), 115.57 (CF_3), 119.22 (NC−C(3′)), 120.66 (NC−C(5)), 126.80 (C(3″)), 128.83 (C(4′)), 130.73 (C(4″)), 131.29 (C(6′)), 132.90 (C(2′)), 135.35 (C(5′)), 137.13 (C(3)), 139.82 (C(5″)), 147.39 (C(2″)), 147.90 (C(1′)), 157.03 (C(7a)), 160.45 (q, ^2J(C,F) = 33.7 Hz, CF_3CO_2), 162.90 (C(6)), 164.06 ppm (CONH). IR (ATR): ν = 3312 (w), 3202 (w), 2969 (w), 2875 (w), 2233 (w), 2187 (w), 1778 (w), 1674 (m), 1633 (s), 1587 (m), 1551 (m), 1523 (m), 1487 (m), 1423 (m), 1392 (m), 1306 (m), 1197 (s), 1171 (s), 1132 (s), 1047 (m), 985 (m), 815 (m), 798 (m), 720 (m), 705 cm^{-1} (s). HR-ESI-MS m/z (%): 573.2469 (37), 572.2438 (100, [M + H]$^+$; calcd for $C_{30}H_{34}N_7O_3S^+$, 572.2438).

(±)-5-[3-(6-Amino-5-cyano-3-methyl-4-isopropyl-2,4-dihydropyrano[2,3-*c*]pyrazol-4-yl)-5-cyanophenyl]-*N*-[3-(4-methylpiperazin-1-yl)propyl]thiophene-2-carboxamide ((±)-5). Following GP1, the active ester of (±)-**9** (0.34 mmol) and 3-(4-methylpiperazino)propylamine (131 mg, 0.83 mmol) were reacted at 25 °C over 20 min. Purification according to PP1 using a 10 times larger volume for all phases (100 mL instead of 10 mL) and conventional separatory funnels because of lower solubility yielded (±)-**5** (154 mg, 77%) contaminated with traces of imidazole, which were separated according to PP2 (eluent H_2O/MeCN 75:25). R_f = 0.24 (Al_2O_3; EtOH/EtOAc 10:90); mp 160−172 °C. ^{1}H NMR (400 MHz, CD_3CN): δ = 0.85 and 0.95 (2 d, J = 6.6 Hz, 6 H; CHMe_2), 1.72 (quint, J = 6.5 Hz, 2 H; $CH_2CH_2CH_2$), 1.80 (s, 3 H; Me−C(3″)), 2.19 (s, 3 H; NMe), 2.31−2.41 (br, s, 8 H; N(CH_2CH_2)$_2$N), 2.45 (t, J = 6.4 Hz, 2 H; N$CH_2CH_2CH_2$), 2.84 (heptet, J = 6.7 Hz, 1 H; CHMe$_2$), 3.39 (td, J = 6.5, 5.2 Hz, 2 H; NHCH_2), 5.47 (br s, 2 H; NH_2), 7.45 (d, J = 3.9 Hz, 1 H; H−C(4)), 7.54 (d, J = 3.9 Hz, 1 H; H−C(3)), 7.75 (t, J = 1.6 Hz, 1 H; H−C(4′)), 7.81 (br t, J = 5.5 Hz, 1 H; CONH), 7.88 (t, J = 1.8 Hz, 1 H; H−C(2′)), 7.94 (t, J = 1.6 Hz, 1 H; H−C(6′)), 10.20−10.80 ppm (br s, 1 H; H−N(2″)). ^{13}C NMR (100 MHz, CD_3CN): δ = 12.12 (Me−C(3″)), 18.63 and 19.08 (CHMe_2), 26.12 ($CH_2CH_2CH_2$), 36.00 (CHMe$_2$), 40.36 (NHCH_2), 46.26 (NMe), 48.58 (C(4″)), 53.98 (CH_2N(CH_2CH_2)$_2$N), 55.82 (NCH_2CH_2NCH$_3$), 57.78 (N-(CH_2CH_2)$_2CH_2$), 63.55 (C(5″)), 99.93 (C(3″a)), 113.99 (C(5′)), 119.26 (NC−C(5′)), 120.62 (NC−C(5″)), 126.46 (C(4)), 128.65 (C(6′)), 129.60 (C(3)), 131.20 (C(2′)), 132.62 (C(4′)), 135.58 (C(1′)), 137.02 (C(3″)), 141.53 (C(2)), 146.08 (C(5)), 147.87 (C(3′)), 157.10 (C(7″a)), 161.95 (CONH), 162.87 ppm (C(6″)). IR (ATR): ν = 3301 (w), 3162 (m), 3087 (w), 2935 (m), 2878 (m), 2805 (m), 2231 (w), 2185 (m), 1632 (s), 1585 (s), 1547 (s), 1520 (s), 1486 (s), 1462 (m), 1421 (m), 1390 (s), 1284 (s), 1148 (s), 1103 (m), 1071 (m), 1048 (m), 1011 (m), 930 (m), 885 (m), 817 (m), 734 cm^{-1} (m). HR-ESI-MS m/z (%): 586.2787 (41), 585.2751 (100, [M + H]$^+$; calcd for $C_{31}H_{37}N_8O_2S^+$, 585.2755).

(4″*R*/*S*)-Di-*tert*-Butyl *N*-[(5-[3-(6-Amino-5-cyano-3-methyl-4-isopropyl-2,4-dihydropyrano[2,3-*c*]pyrazol-4-yl)-5-cyanophenyl]thien-2-yl)carbonyl]-L-glutamate ((4″*R*/*S*,2‴*S*)-6). Following GP1, the active ester of (±)-**9** (0.34 mmol) and **29** (302 mg, 1.02 mmol) were reacted. Addition of *i*-Pr$_2$NEt (352 mg, 2.72 mmol) to dissolve the formed precipitate and a reaction time of 3 h at 60 °C were required for completion of the reaction. Chromatographic purification on a Teledyne CombiFlash MPLC system (RediSep 40 g SiO_2, dry loading, EtOAc/cyclohexane 50:50) yielded pure (4″*R*/*S*,2‴*S*)-**6** as a white solid (108 mg, 46%). R_f = 0.44 (SiO_2, EtOAc/cyclohexane 70:30); mp 133−145 °C. ^{1}H NMR (400 MHz, (D_8)THF): δ = 0.89 and 0.99 (2 d, J = 6.6 Hz, 6 H; CHMe_2), 1.42 (s, 9 H; CMe$_3$), 1.46 (s, 9 H; CMe$_3$), 1.87 (s, 3 H; Me−C(3″)), 1.88−1.98 (m, 1 H; CHCH_aH$_b$), 2.10−2.20 (m, 1 H; CHCH$_a$$H_b$), 2.30−2.38 (m, 2 H; CH_2CO_2), 2.88 (heptet, J = 6.7 Hz, 1 H; CHMe$_2$), 4.50−4.58 (br td, J = 8.2, 4.4 Hz, 1 H; NHCH), 6.29 (s, 2 H; NH_2), 7.47 (d, J = 3.9 Hz, 1 H; H−C(4)), 7.64 (d, J = 3.9 Hz, 1 H; H−C(3)), 7.69 (br t, J = 1.6 Hz, 1 H; H−C(4′)), 7.72 (br d, J = 8.3 Hz, 1 H; CONH), 7.92 (br t, J = 1.8 Hz, 1 H; H−C(2′)), 7.95 (br t, J = 1.5 Hz, 1 H; H−C(6′)), 11.35 ppm (s, 1 H; NNH). ^{13}C NMR (100 MHz, (D_8)THF): δ = 12.32 (Me−C(3″)), 18.93 and 19.44 (CHMe_2), 28.32 (CHCH$_2$), 28.36 (OCMe_3), 28.45 (OCMe_3), 32.60 (CH_2CO_2), 36.30 (CHMe$_2$), 48.90 (C(4″)), 53.73 (NHCH), 62.81 (C(5″)), 80.66 (OCMe$_3$), 81.90 (OCMe$_3$), 99.68 (C(3″a)), 114.56 (C(5′)), 119.12

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(NC−C(5′)), 120.12 (NC−C(5″)), 125.95 (C(4)), 128.23 (C(6′)), 129.80 (C(3)), 131.00 (C(2′)), 132.61 (C(4′)), 135.92 and 135.98 (C(1′, 3″)), 141.25 (C(2)), 147.20 (C(5)), 148.37 (C(3′)), 157.53 (C(7″a)), 161.87 (CONH), 162.99 (C(6″)), 171.97 ($CHCO_2$), 172.60 ppm (CH_2CO_2). IR (ATR): ν = 3315 (w), 2975 (w), 2932 (w), 2232 (w), 2187 (w), 1725 (m), 1632 (s), 1587 (m), 1544 (m), 1517 (m), 1487 (m), 1421 (m), 1392 (m), 1367 (s), 1292 (m), 1249 (m), 1148 (s), 1070 (m), 1035 (m), 907 (w), 843 (m), 819 (m), 734 cm^{-1} (m). HR-ESI-MS m/z (%): 710.2822 (34), 709.2787 (68, $[M + Na]^+$; calcd for $C_{36}H_{42}N_6NaO_6S^+$, 709.2779), 575.1713 (100).

(4*R*/*S*)-5-Cyano-4-(3-cyano-5-{5-[((*S*)-1,3-dicarboxypropyl)-carbamoyl]thiophen-2-yl}phenyl)-3-methyl-4-(propan-2-yl)-2,4-dihydropyrano[2,3-*c*]pyrazol-6-aminium Trifluoroacetate ((4*R*/*S*,2‴*S*)-7). A solution of (4″*R*/*S*,2‴*S*)-**6** (19 mg, 0.028 mmol) in TFA (1 mL) was kept in a flask sealed with a glass stopper at 25 °C for 14 h. Excess TFA was distilled off. The residue was dried under high vacuum to yield pure (4*R*/*S*,2‴*S*)-7 (19 mg, quant) as a yellow solid. Prolonged exposure to high vacuum may vary the amount of trifluoroacetate. The precise content was determined by ^1H and ^{19}F NMR integration and was found to be 0.8 equiv of TFA. Dibromofluoromethane was used as an internal standard to correlate ^1H and ^{19}F integral ratios. Compound (4*R*/*S*,2‴*S*)-7 may be converted to the hydrochloride salt (4*R*/*S*,2‴*S*)-**7a** by dissolving in THF, adding excess aqueous HCl solution (1 N), followed by concentrating and drying under vacuum. Data of (4*R*/*S*,2‴*S*)-7: R_f = 0.32−0.47 (SiO_2; H_2O/THF 5:95); mp 123−131 °C. ^1H NMR (400 MHz, (D_8)THF): δ = 0.89 and 0.99 (2 d, J = 6.6 Hz, 6 H; CHMe_2), 1.86 (s, 3 H; Me−C(3)), 1.94−2.06 (m, 1 H; CHCH_aH_b), 2.17−2.28 (m, 1 H; CHCH_aH_b), 2.33−2.48 (m, 2 H; CH_2CO_2), 2.88 (heptet, J = 6.7 Hz, 1 H; CHMe$_2$), 4.63−4.70 (br td, J = 8.6, 4.8 Hz, 1 H; NHCH), 6.29 (br s, 2 H; NH_2), 7.47 (d, J = 3.9 Hz, 1 H; H−C(4″)), 7.64 (d, J = 3.9 Hz, 1 H; H−C(3″)), 7.69 (br t, J = 1.6 Hz, 1 H; H−C(2′)), 7.78 (br d, J = 8.3 Hz, 1 H; CONH), 7.91 (br t, J = 1.8 Hz, 1 H; H−C(6′)), 7.95 ppm (br t, J = 1.5 Hz, 1 H; H−C(4′)). ^{13}C NMR (100 MHz, (D_8)THF): δ = 12.33 (Me−C(3)), 18.92 and 19.44 (CHMe_2), 28.30 ($CHCH_2$), 31.04 (CH_2CO_2), 36.30 (CHMe$_2$), 48.90 (C(4)), 52.91 (NHCH), 62.80 (C(5)), 99.67 (C(3a)), 114.56 (C(3′)), 119.12 (NC−C(3′)), 120.13 (NC−C(5)), 125.94 (C(4″)), 128.22 (C(4′)), 129.77 (C(3″)), 131.01 (C(6′)), 132.59 (C(2′)), 135.95 and 136.01 (C(5′, 3)), 141.36 (C(2″)), 147.13 (C(5″)), 148.36 (C(1′)), 157.52 (C(7a)), 161.82 (CONH), 163.00 (C(6)), 173.61 ($CHCO_2$), 174.48 ppm (CH_2CO_2). IR (ATR): ν = 3500−2200 (m), 3313 (w), 3187 (w), 3094 (w), 2967 (w), 2931 (w), 2234 (w), 2188 (w), 1714 (m), 1632 (s), 1587 (s), 1545 (s), 1519 (s), 1493 (m), 1421 (m), 1392 (s), 1347 (m), 1296 (m), 1259 (s), 1203 (s), 1149 (s), 1096 (s), 1070 (m), 1030 (s), 935 (m), 883 (m), 816 (s), 740 (m), 695 (m), 656 cm^{-1} (m). HR-ESI-MS m/z (%): 576.1736 (13), 575.1709 (36, $[M + H]^+$; calcd for $C_{28}H_{27}N_6O_6S^+$, 575.1707), 282.2799 (100). Data of (4*R*/*S*,2‴*S*)-7**a**: mp 220−250 °C (dec). IR (ATR): ν = 3310 (m), 3185 (m), 3089 (w), 2964 (m), 2929 (m), 2877 (w), 2548 (w), 2234 (w), 2188 (w), 1710 (m), 1630 (s), 1586 (s), 1546 (s), 1520 (s), 1492 (m), 1421 (s), 1393 (s), 1295 (s), 1259 (s), 1213 (s), 1175 (s), 1153 (s), 1095 (m), 1033 (s), 881 (m), 818 (s), 745 (m), 694 (m), 651 cm^{-1} (m).

(±)-5-[3-(6-Amino-5-cyano-3-methyl-4-isopropyl-2,4-dihydropyrano[2,3-*c*]pyrazol-4-yl)-5-cyanophenyl]thiophene-2-carboxylic Acid ((±)-9). A solution of (±)-**8** (500 mg, 0.93 mmol) in MeOH (10 mL) was treated with 10% Pd/C (250 mg) and stirred under H_2 (two balloons (⌀ 25 cm) of H_2 were passed through the solution) for 30 min. The solid was filtered off and the filtrate evaporated. The residue was suspended in EtOAc (50 mL) by sonication and extracted twice with aqueous NH_3 solution (50 mL, pH 11). The aqueous phase was acidified to pH 5 with 1 N HCl and extracted with EtOAc (3 × 150 mL). The precipitate should rapidly be dissolved in the organic phase to avoid aggregation. The organic phase was washed with aqueous saturated NaCl solution (250 mL) and evaporated. Drying under high vacuum for 2 days gave (±)-**9** (367 mg, 88%) as a white powder: mp 250−270 °C (dec). ^1H NMR (400 MHz, (D_8)THF): δ = 0.89 and 0.99 (2 d, J = 6.6 Hz, 6 H; CHMe_2), 1.87 (s, 3 H; Me−C(3″)), 2.89 (heptet, J = 6.5 Hz, 1 H; CHMe$_2$), 6.30 (br s, 2 H; NH_2), 7.51 (d, J = 3.9 Hz, 1 H; H−C(4)), 7.71 (d, J = 3.9 Hz, 1 H; H−C(3)), 7.72 (t, J = 1.5 Hz, 1 H; H−C(4′)), 7.94 (t, J = 1.8 Hz, 1 H; H−C(2′)), 7.98 (t, J = 1.5 Hz, 1 H; H−C(6′)), 10.8−11.8 ppm (br s, 2 H; COOH, NH). ^{13}C NMR (100 MHz, (D_8)THF): δ = 12.32 (CH_3−C(3″)), 18.92 and 19.44 (CHMe_2), 36.30 (CHMe$_2$), 48.92 (C(4″)), 62.78 (C(5″)), 99.65 (C(3″a)), 114.63 (C(5′)), 119.05 (NC−C(5′)), 120.12 (NC−C(5″)), 126.31 (C(4)), 128.46 (C(6′)), 131.10 (C(2′)), 132.96 (C(4′)), 135.03 (C(3)), 135.68 and 135.91 and 135.98 (C(2, 1′, 3″)), 148.45 (C(3′)), 149.16 (C(5)), 157.53 (C(7″a)), 163.00 and 163.02 ppm (CO_2, C(6″)). IR (ATR): ν = 3494 (w), 3390 (w), 3341 (w), 3200 (w), 3166 (w), 3097 (w), 2966 (w), 2875 (w), 2236 (w), 2202 (m), 1704 (s), 1635 (s), 1590 (s), 1536 (w), 1487 (m), 1468 (m), 1430 (m), 1388 (s), 1337 (w), 1279 (m), 1249 (s), 1213 (m), 1190 (m), 1156 (m), 1102 (m), 1067 (w), 1046 (m), 1024 (m), 932 (m), 909 (w), 869 (w), 839 (m), 794 (w), 751 (m), 732 (s), 706 cm^{-1} (m). HR-ESI-MS m/z (%): 447.1313 (29), 446.1283 (100, $[M + H]^+$; calcd for $C_{23}H_{20}N_5O_3S^+$, 446.1281).

5-[3-(6-Amino-5-cyano-4-isopropyl-3-methyl-2,4-dihydropyrano[2,3-*c*]pyrazol-4-yl)-5-cyanophenyl]-*N*,*N*-dimethyl-2-thiophenecarboxamide ((±)-10). Following GP1, dimethylamine (2 M in THF) (0.20 mL, 0.39 mmol) was added to the active ester of (±)-**9** (56 mg, 0.11 mmol) in THF (4.9 mL). The mixture was stirred at 25 °C for 16 h. The mixture was concentrated under reduced pressure. Chromatography (SiO_2, gradient EtOAc/CH_2Cl_2 50:50 to 60:40) yielded the product (±)-**10** as a white solid (35 mg, 66%). R_f = 0.12 (SiO_2, gradient EtOAc/CH_2Cl_2 60:40); mp 166−169 °C. ^1H NMR (400 MHz, (D_8)THF): δ = 11.35 (s, 1H; NNH), 7.95 (br t, J = 1.6 Hz, 1H; H−C(6′)), 7.92 (br t, J = 1.8 Hz, 1H; H−C(2′)), 7.69 (br t, J = 1.6 Hz, 1H; H−C(4′)), 7.44 (d, J = 3.9 Hz, 1H; H−C(3)), 7.39 (d, J = 3.9 Hz, 1H; H−C(4)), 6.28 (s, 2H; NH_2), 3.14 (s, 6H; NMe_2), 2.88 (p, J = 6.6 Hz, 1H; CHMe$_2$), 1.86 (s, 3H; Me−C(3″)), 0.98 (d, J = 6.6 Hz, 3H; CHMe_2), 0.88 (d, J = 6.6 Hz, 3H; CHMe_2) ppm. ^{13}C NMR (125 MHz, (D_8)THF): δ = 163.55 (C(6″)), 162.99 (CONMe$_2$), 157.54 (C(7″a)), 148.36 (C(5)), 145.88 (C(3′)), 140.87 (C(3″)), 135.96, 135.87 (C(2)), 132.52 (C(3)), 130.98 (C(4′)), 130.92 (C(2′)), 128.18 (C(6′)), 125.30 (C(4)), 120.14 (NC−(5″)), 119.14 (NC−(5′)), 114.52 (C(5′)), 99.70 (C(3″a)), 62.83 (C(5″)), 48.89 (C(4″)), 36.30 (NMe_2), 19.45 (CHMe_2), 18.94 (CHMe_2), 12.32 (Me−C(3″)) ppm, CHMe$_2$ obscured by solvent residual signal of (D_8)THF. IR (ATR): ν = 3175 (br w), 2967 (w), 2932 (w), 2231 (w), 2185 (m), 1632 (s), 1585 (s), 1538 (w), 1487 (m), 1435 (w), 1420 (w), 1390 (s), 1284 (w), 1265 (w), 1204 (w), 1183 (w), 1152 (w), 1057 (br m), 877 (br w), 813 (m), 733 (m), 695 (m). HR-ESI-MS m/z (%): 473.1748 (100, $[M + H]^+$; calcd for $C_{25}H_{25}N_6O_2S^+$, 473.1754). Elemental analysis calcd (%) for $C_{25}H_{24}N_6O_2S$: C, 63.54; H, 5.12; N, 17.78. Found: C, 62.10; H, 5.37; N, 16.55.

Safety Precautions. Diazomethane and its derivatives are explosive and highly toxic. Experiments were carried out behind a blast shield in a dedicated laboratory. Phenyldiazomethane was quantified by ^1H NMR measurements ($CDCl_3$, δ = 4.94 ppm (s, 1 H; PhCHN$_2$)).

Benzyl 5-(4,4,5,5-Tetramethyl-1,3,2-dioxaborolan-2-yl)-thiophene-2-carboxylate (11). A solution of Me_2SO (3.84 g, 49.3 mmol, 2.2 equiv) in Et_2O/CH_2Cl_2 9:1 (470 mL) was cooled to −60 °C in an argon filled 2 L flask, treated dropwise with oxalyl chloride (5.97 g, 47 mmol), and stirred for 20 min after which gas evolution had ceased. A solution of Et_3N (9.50 g, 94.1 mmol) and **27** (5.38 g, 44.8 mmol) in Et_2O/CH_2Cl_2 9:1 (150 mL) was added dropwise to the cold mixture behind a blast shield over 20 min.[26] A precipitate was formed, and the mixture turned "peach" colored. The mixture was stirred for 50 min at −60 °C, treated dropwise with a solution of **26** (5.69 g, 22.4 mmol) in Et_2O/CH_2Cl_2 9:1 (75 mL) over 20 min, and stirred in the dark for 14 h at 25 °C. Excess phenyldiazomethane **68** was deleted by addition of a 1.75 N solution of acetic acid in Et_2O. The mixture was treated with SiO_2 and evaporated. Chromatography (Teledyne CombiFlash MPLC system, RediSep 120 g SiO_2, dry loading, EtOAc/cyclohexane 2:98) yielded the product **11** (6.73 g, 87%) as a yellow/orange oil which crystallized over 14 h. R_f = 0.53 (SiO_2; EtOAc/cyclohexane 20:80); mp 79−80 °C. ^1H NMR (400 MHz, (D_8)THF): δ = 1.32 (s, 12 H; $(CMe_2)_2$), 5.31 (s, 2 H; CH_2Ph), 7.26−7.37 (m, 3 H; H−C(3′, 4′, 5′)), 7.50−7.53 (br d, J = 7.4 Hz 2 H; H−C(2′, 6′)), 7.52 (d, J = 3.7 Hz, 1 H; H−C(4)), 7.81 ppm (d, J = 3.7 Hz, 1 H; H−C(3)); ^{13}C NMR (100

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MHz, (D_8)THF): δ = 25.21 ((CMe_2)$_2$), 67.44 (CH_2Ph), 85.44 ((CMe_2)$_2$), 129.01 (C(2′, 6′)), 129.03 (C(4′)), 129.40 (C(3′, 5′)), 134.71 (C(3)), 137.47 (C(1′)), 137.83 (C(4)), 140.78 (C(2)), 162.08 ppm (C═O), C(5) hidden by the noise. IR (ATR): ν = 2976 (w), 1713 (s), 1525 (m), 1471 (w), 1455 (w), 1384 (m), 1358 (s), 1329 (s), 1292 (s), 1235 (s), 1142 (s), 1087 (s), 1073 (s), 1057 (s), 1019 (m), 961 (m), 919 (m), 851 (s), 824 (m), 783 (w), 740 cm^{-1} (s). HR-ESI-MS m/z (%): 368.1181 (9), 367.1151 (43, [M + H]$^+$; calcd for $C_{18}H_{21}{}^{11}BNaO_4S^+$, 367.1149), 366.1185 (9, [M + H]$^+$; calcd for $C_{18}H_{21}{}^{10}BNaO_4S^+$, 366.1182), 359.0181 (100).

3-Bromo-5-(2-methylpropanoyl)benzonitrile (12). To a solution of 3,5-dibromobenzonitrile (10.95 g, 42.0 mmol) in dry THF (313 mL) at 0 °C was added dropwise isopropylmagnesium chloride in Et_2O (20.98 mL of a 2 M solution, 42.0 mmol). A brown solution was obtained. The mixture was stirred at 25 °C for 1 h. A solution of **24** (6.06 g, 46.2 mmol) in THF (21 mL) was added dropwise at 0 °C. The mixture was stirred at 25 °C for 15 h, treated with saturated aqueous NH_4Cl (75 mL) solution, and diluted with EtOAc (75 mL). The aqueous layer was extracted with EtOAc (75 mL). The combined organic layers were washed with H_2O (75 mL), saturated aqueous NaCl (75 mL), dried over Na_2SO_4, filtered, and evaporated to afford an orange oil containing a white solid. The mixture was suspended in cyclohexane and filtered. The filtrate was evaporated and the residue subjected to chromatography (SiO_2, gradient EtOAc/cyclohexane 0:100 to 02:98). Product **12** was obtained as a faint yellow oil (6.10 g, 58%). R_f = 0.30 (SiO_2; cyclohexane/EtOAc 95:05). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.22 (d, J = 6.9 Hz, 6 H; CHMe_2), 3.45 (heptet, J = 6.8 Hz, 1 H; CHMe$_2$), 7.95 (br t, J = 1.66 Hz, 1 H; H−C(2), 8.13 (br t, J = 1.47 Hz, 1 H; H−C(6)), 8.27 ppm (br t, J = 1.73 Hz, 1 H; H−C(4)). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 18.92 (CHMe_2), 35.95 (CHMe$_2$), 114.88 (C(1)), 116.72 (CN), 123.79 (C(3)), 130.54 (C(6)), 135.69 (C(4)), 138.20 (C(2)), 138.54 (C(5)), 201.02 ppm (C═O). IR (ATR): ν = 3073 (w), 2973 (w), 2933 (w), 2873 (w), 2234 (w), 1691 (s), 1592 (w), 1563 (m), 1466 (m), 1420 (m), 1384 (m), 1351 (m), 1279 (m), 1235 (s), 1187 (m), 1141 (m), 1085 (m), 1031 (m), 1018 (s), 965 (w), 920 (w), 882 (s), 851 (m), 832 (m), 770 (m), 746 (m), 669 (s), 608 cm^{-1} (w). HR-EI-MS m/z (%): 250.9939 (18, [M$^+$]; calcd for $C_{11}H_{10}BrNO^+$, 250.9941).

Benzyl 5-[3-Cyano-5-(2-methylpropanoyl)phenyl]-thiophene-2-carboxylate (13). A solution of **12** (4.20 g, 16.7 mmol), **11** (5.76 g, 16.7 mmol), and Na_2CO_3 (2.12 g, 20 mmol) in THF/H_2O 80:20 (250 mL) was degassed by sonication while passing argon through the solution for 5 min, treated with [Pd(PPh_3)$_2$Cl$_2$] (290 mg, 0.42 mmol), and degassed by sonication under argon for 10 min. The mixture was heated to 60 °C for 4 h, diluted with EtOAc, and washed with saturated aqueous NaCl solution. The organic phase was evaporated, and the residue was adsorbed on 20 g of SiO_2. Column chromatography on a Teledyne CombiFlash MPLC system (RediSep 120 g SiO_2, dry loading, EtOAc/cyclohexane 5:95) yielded pure **13** (5.09 g, 78%) as a yellow oil which solidified upon high vacuum drying. R_f = 0.36 (SiO_2; EtOAc/cyclohexane 20:80); mp 100−101 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.26 (d, J = 6.8 Hz, 6 H; CHMe_2), 3.52 (heptet, J = 6.8 Hz, 1 H; CHMe$_2$), 5.37 (s, 2 H; CH_2Ph), 7.41 (d, J = 3.7 Hz, 1 H; H−C(4)), 7.33−7.48 (m, 5 H; Ph), 7.84 (d, J = 3.9 Hz, 1 H; H−C(3)), 8.04 (br t, J = 1.62 Hz, 1 H; H−C(2′)), 8.14 (br t, J = 1.45 Hz, 1 H; H−C(4′)), 8.36 ppm (br t, J = 1.66 Hz, 1 H; H−C(6′)). ^{13}C NMR (100 MHz, $CDCl_3$)): δ = 19.02 (CHMe_2), 35.99 (CHMe$_2$), 67.28 (CH_2Ph), 114.33 (C(3′)), 117.61 (CN), 125.68 (C(4)), 128.40 (C(2″,6″)), 128.62 (C(4″)), 128.82 (C(3″,5″)), 129.79 (C(6′)), 131.39 (C(4′)), 132.85 (C(2′)), 134.56 (C(2)), 134.78 (C(3)), 135.64 and 135.68 (C(1″, 1′)), 137.99 (C(5′)), 147.17 (C(5)), 161.61 (CO_2), 201.84 ppm (C═O). IR (ATR): ν = 3073 (w), 2977 (w), 2933 (w), 2871 (w), 2232 (w), 1687 (s), 1588 (w), 1532 (w), 1499 (w), 1458 (m), 1441 (m), 1423 (m), 1379 (m), 1357 (w), 1282 (s), 1222 (m), 1195 (m), 1149 (m), 1095 (s), 1073 (s), 1040 (s), 996 (m), 914 (m), 901 (m), 884 (m), 817 (m), 746 cm^{-1} (s). HR-ESI-MS m/z (%): 407.1426 (100), 391.1196 (18), 390.1160 (62, [M + H]$^+$; calcd for $C_{23}H_{20}NO_3S^+$, 309.1158).

Benzyl 5-[3-Cyano-5-(1,1-dicyano-3-methylbut-1-en-2-yl)-phenyl]thiophene-2-carboxylate (14). A solution of **13** (2.76 g, 7.09 mmol) and malononitrile (2.34 g, 35.5 mmol) in unstabilized dry $CHCl_3$ (20 mL) was treated with a solution of 1 M $TiCl_4$ in toluene (9.2 mL, 9.2 mmol) leading to a light brown color and precipitation. Pyridine (2.3 mL, 28.4 mmol) was added leading to a darkening. The mixture was stirred at 60 °C for 4 days and diluted with MeOH (20 mL) to get a solution. The mixture was treated with SiO_2 (20 g) and evaporated. Chromatography on a Teledyne CombiFlash MPLC system (RediSep 80 g SiO_2, dry loading, EtOAc/cyclohexane 10:90) yielded **14** (2.11 g, 68%) as a green tinted solid. R_f = 0.27 (SiO_2, EtOAc/cyclohexane 20:80); mp 128−129 °C. ^{1}H NMR (400 MHz, $CDCl_3$)): δ = 1.22 (d, J = 6.9 Hz, 6 H; CHMe_2), 3.52 (heptet, J = 6.9 Hz, 1 H; CHMe$_2$), 5.37 (s, 2 H; CH_2Ph), 7.38 (d, J = 4.0 Hz, 1 H; H−C(4)), 7.42 (br t, J = 1.5 Hz, 1 H; H−C(4′)), 7.33−7.48 (m, 5 H; Ph), 7.59 (br t, J = 1.7 Hz, 1 H; H−C(6′)), 7.84 (d, J = 3.9 Hz, 1 H; H−C(3)), 8.01 ppm (br t, J = 1.6 Hz, 1 H; H−C(2′)). ^{13}C NMR (100 MHz, $CDCl_3$)): δ = 20.67 (CHMe_2), 36.24 (CHMe$_2$), 67.38 (CH_2Ph), 89.01 (C(CN)$_2$), 110.85 and 111.29 (C(CN)$_2$), 114.81 (C(3′)), 117.04 (NC−C(3′)), 126.09 (C(4)), 128.45 (C(6′), C(2, 6) of Ph), 128.66 (C(4) of Ph), 128.82 (C(3,5) of Ph), 129.57 (C(4′)), 131.11 (C(2′)), 134.79 (C(3)), 135.10 (C(2)), 135.54 (C(1) of Ph), 136.02 and 136.13 (C(1′, 5′)), 146.24 (C(5)), 161.47 (CO_2), 182.68 ppm (C═C(CN)$_2$). ^{1}H NMR (300 MHz, (D_8)THF): δ = 1.21 (d, J = 6.9 Hz, 6 H; CHMe_2), 3.47 (heptet, J = 6.9 Hz, 1 H; CHMe$_2$), 5.33 (s, 2 H; CH_2Ph), 7.28−7.40 (m, 3 H of Ph), 7.41−7.48 (m, 2 H of Ph), 7.66 (d, J = 4.0 Hz, 1 H; H−C(4)), 7.72 (br t, J = 1.5 Hz, 1 H; H−C(4′)), 7.84 (d, J = 4.0 Hz, 1 H; H−C(3)), 7.91 (br t, J = 1.7 Hz, 1 H; H−C(6′)), 8.33 ppm (br t, J = 1.6 Hz, 1 H; H−C(2′)). ^{13}C NMR (75 MHz, (D_8)THF): δ = 20.60 (CHMe_2), 37.18 (CHMe$_2$), 67.78 (CH_2Ph), 89.70 (C(CN)$_2$), 112.27 and 112.62 (C(CN)$_2$), 115.58 (C(3′)), 118.08 (NC−C(3′)), 127.30 (C(4)), 129.21 (C(4) of Ph), 129.31 (C(2,6) of Ph), 129.47 (C(3,5) of Ph), 129.65 (C(6′)), 131.26 (C(4′)), 131.87 (C(2′)), 135.48 (C(3)), 135.62 (C(2)), 136.47 (C(1′)), 137.27 (C(1) of Ph), 137.57 (C(5′)), 147.92 (C(5)), 161.89 (C═O), 183.04 (C═C(CN)$_2$) ppm. IR (ATR): ν = 3116 (w), 3071 (w), 2978 (w), 2939 (w), 2232 (w), 1707 (s), 1698 (s), 1592 (m), 1568 (w), 1539 (m), 1497 (w), 1463 (m), 1419 (m), 1388 (w), 1370 (m), 1348 (m), 1306 (m), 1270 (s), 1254 (s), 1238 (s), 1215 (s), 1158 (w), 1100 (s), 1031 (w), 1014 (w), 965 (m), 905 (m), 886 (m), 858 (m), 824 (m), 742 cm^{-1} (s). HR-ESI-MS m/z (%): 389.1317 (100), 461.1122 (4), 460.1092 (12, [M + H]$^+$; calcd for $C_{26}H_{19}N_3NaO_2S^+$, 460.1090).

(±)-Benzyl 4-[3-(6-Amino-5-cyano-4-isopropyl-3-methyl-1,4-dihydropyrano[2,3-*c*]pyrazol-4-yl)-5-cyanophenyl]piperazine-1-carboxylate ((±)-22). A mixture of **34** (250 mg, 0.57 mmol), 3-methyl-1*H*-pyrazol-5(4*H*)-one (**15**) (56 mg, 0.57 mmol), and piperidine (0.56 mL, 5.7 mmol) in EtOH (2 mL) was stirred for 4 h at 70 °C in a sealed tube in a microwave oven and evaporated. Column chromatography (SiO_2; Et_2O → Et_2O/THF 1:1) gave (±)-**22** (120 mg, 39%) as an off-white solid. R_f = 0.39 (SiO_2; Et_2O/THF 2:1): mp 118−119 °C. ^{1}H NMR (400 MHz, CD_3OD): δ = 0.86 and 0.98 (2 d, J = 6.6 Hz, 6 H; CHMe_2), 1.83 (s, 3 H; Me−C(3′)), 2.80 (heptet, J = 6.5 Hz, 1 H; CHMe$_2$), 3.19 (br t, J = 5.2 Hz, 4 H; (CH$_2$)$_2$N−Ar), 3.64 (br s, 4 H; (CH$_2$)$_2$N−CO), 5.14 (s, 2 H; CH$_2$Ph), 7.18 (br d, $J \approx$ 1.9, 2 H; H−C(2,4)), 7.26 (br t, J = 2.1, 1 H; H−C(2)), 7.28−7.41 ppm (m, 5 H; Ph). ^{13}C NMR (100 MHz, CD_3OD): δ = 11.91 (Me−C(3′)), 18.80 and 19.26 (CHMe_2), 36.52 (CHMe$_2$), 44.69 (br, (CH$_2$)$_2$N−CO), 48.96 (C(4′)), 49.78 ((CH$_2$)$_2$N−Ar), 62.05 (C(5′)), 68.46 (CH$_2$Ph), 100.84 (C(3′a)), 113.73 (C(5)), 118.51 (C(6)), 120.19 (NC−C(5)), 121.76 (NC−C(5′)), 122.88 (C(2)), 124.41 (C(4)), 128.96 (C(2,6) of Ph), 129.17 (C(4) of Ph), 129.56 (C(3,5) of Ph), 137.49 (C(3′)), 138.01 (C(1) of Ph), 148.01 (C(3)), 153.04 (C(1)), 156.85 (CO_2N), 157.45 (C(7′a)), 163.95 ppm (C(6′)). IR (ATR): $\tilde{\nu}$ = 3339 (w), 3161 (w), 2958 (w), 2228 (w), 2191 (m), 1703 (m), 1656 (m), 1584 (s), 1490 (m), 1430 (s), 1399 (m), 1361 (m), 1288 (m), 1239 (s), 1223 (s), 1158 (m), 1123 (m), 1078 (m), 1039 (m), 997 (s), 902 (m), 860 (m), 758 (m), 729 (s), 695 (m), 673 cm^{-1} (m). HR-MALDI-MS: m/z (%): 560.2379 (100, [M + Na]$^+$; calcd for $C_{30}H_{31}N_7NaO_3^+$, 560.2381).

(±)-6-Amino-4-[3-cyano-5-(piperazin-1-yl)phenyl]-4-isopropyl-3-methyl-1,4-dihydropyrano[2,3-*c*]pyrazole-5-carbonitrile ((±)-23). A suspension of (±)-**22** (80 mg, 0.15 mmol) and Pd/C (10% Pd, 10 mg) in MeOH (2 mL) was purged with N_2 for 30 min, stirred at 25 °C under an H_2 atmosphere for 16 h, and filtered through a pad of Celite. Evaporation yielded (±)-**23** (57 mg, 94%) as an orange

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oil: mp > 191 °C (dec). ^{1}H NMR (400 MHz, CD_3OD): δ = 0.86 and 0.99 (2 d, J = 6.6 Hz, 6 H; CHMe_2), 1.84 (s, 3 H; Me−C(3)), 2.82 (heptet, J = 6.5 Hz, 1 H, CHMe$_2$), 3.31−3.36 (m, 4 H; $(CH_2)_2$N−Ar)), 3.40−3.45 (m, 4 H; $(CH_2)_2$N−CO)), 7.27 (br dd, J = 2.4, 1.3 Hz, 1 H; H−C(4′)), 7.28 (br t, J = 1.4 Hz, 1 H; H−C(2′)), 7.32 ppm (br dd, J = 2.4, 1.7 Hz, 1 H; H−C(6′)). ^{13}C NMR (100 MHz, CD_3OD): δ = 11.88 (Me−C(3)), 18.78 and 19.23 (CHMe_2), 36.54 (CHMe$_2$), 44.73 ($(CH_2)_2$NH), 47.55 ($(CH_2)_2$N−Ar), 49.03 (C(4)), 61.88 (C(5)), 100.76 (C(3a)), 113.94 (C(3′)), 118.93 (C(4′)), 119.96 (NC−C(3′)), 121.81 (NC−C(5)), 123.28 (C(6′)), 125.35 (C(2′)), 137.47 (C(3)), 148.34 (C(1′)), 152.27 (C(5′)), 157.41 (C(7a)), 164.02 (C(6)). IR (ATR): $\tilde{\nu}$ = 3100 (w), 2184 (w), 1626 (m), 1576 (s), 1380 (m), 1242 (s), 1148 (s), 996 cm^{-1} (m). HR-MALDI-MS m/z (%): 404.2193 (100, $[M + H]^+$; calcd for $C_{22}H_{26}N_7O^+$, 404.2193).

Methyl 5-(3-Cyano-5-isobutyrylphenyl)thiophene-2-carboxylate (31). A mixture of $Pd(OAc)_2$ (47 mg, 0.21 mmol) and S-Phos (172 mg, 0.42 mmol) in degassed THF (7 mL) was stirred under N_2 at 25 °C for 30 min. 3-Bromo-5-isobutyrylbenzonitrile **12** (1.765 g, 7.0 mmol) and **30** (1.475 g, 7.8 mmol) were added, and the mixture was combined with a solution of K_2CO_3 (2.900 g, 21 mmol) in degassed water (3 mL) and stirred at 45 °C for 16 h. The mixture was diluted with water (100 mL) and EtOAc (100 mL), and the layers were separated. The organic phase was dried over Na_2SO_4 and evaporated. The crude product was triturated with i-Pr_2O to yield **31** (1.860 g, 85%) as a gray powder. Mp 139−140 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.26 (d, J = 6.9 Hz, 6 H; CHMe_2), 3.52 (septet, J = 6.9 Hz, 1 H; CHMe$_2$), 3.93 (s, 3 H; OMe), 7.40 (d, J = 3.9 Hz, 1 H; H−C(4)), 7.80 (d, J = 3.9 Hz, 1 H; H−C(3)), 8.04 (t, J = 1.6 Hz, 1 H; H−C(2′)), 8.14 (t, J = 1.5 Hz, 1 H; H−C(4′)), 8.36 ppm (t, J = 1.7 Hz, 1 H; H−C(6′)). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 19.00 (CHMe_2), 35.97 (CHMe$_2$), 52.59 (OMe), 114.29 (C(3′)), 117.61 (CN), 125.66 (C(4)), 129.78 (C(6′)), 131.36 (C(4′)), 132.83 (C(2′)), 134.50 (C(2)), 134.57 (C(3)), 135.67 (C(1′)), 137.96 (C(5′)), 146.97 (C(5)), 162.22 (CO_2), 201.82 (C=O) ppm. IR (ATR): $\tilde{\nu}$ = 3098 (w), 2972 (w), 2237 (w), 1707 (s), 1684 (m), 1594 (w), 1538 (w), 1469 (w), 1425 (m), 1381 (w), 1351 (w), 1316 (w), 1291 (m), 1270 (m), 1254 (s), 1227 (m), 1199 (m), 1186 (m), 1150 (w), 1095 (s), 1033 (w), 1008 (m), 959 (w), 910 (w), 886 (w), 832 (w), 790 (w), 767 (w), 745 (s), 675 (w), 649 (w), 622 cm^{-1} (m); HR-MALDI-MS: m/z (%): 314.0845 (100, $[M + H]^+$; calcd for $C_{17}H_{16}NO_3S^+$, 314.0845).

Methyl 5-[3-Cyano-5-(1,1-dicyano-3-methylbut-1-en-2-yl)-phenyl]thiophene-2-carboxylate (32). Under N_2, a solution of **31** (625 mg, 2.0 mmol) and malononitrile (528 mg, 8 mmol) in $CHCl_3$ (5 mL) was treated with a 1 M solution of $TiCl_4$ in toluene (2.2 mL, 2.2 mmol). Pyridine (0.49 mL, 6 mmol) was added, and the mixture was stirred at 70 °C for 18 h in a sealed tube. 1 M aqueous HCl (100 mL) and $CHCl_3$ (100 mL) were added. The solid precipitate was filtered off, and the layers were separated. The aqueous phase was extracted with $CHCl_3$ (3 × 50 mL). The combined organic phases were dried over Na_2SO_4 and evaporated. The residue was triturated with i-Pr_2O to yield **32** (656 mg, 91%) as a red solid: mp 189−190 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.22 (d, J = 6.9 Hz, 6 H; CHMe_2), 3.52 (septet, J = 6.9 Hz, 1 H; CHMe$_2$), 3.93 (s, 3 H; OMe), 7.38 (d, J = 3.9 Hz, 1 H; H−C(4)), 7.43 (t, J = 1.5 Hz, 1 H; H−C(4′)), 7.60 (t, J = 1.7 Hz, 1 H; H−C(6′)), 7.80 (d, J = 3.9 Hz, 1 H; H−C(3)), 8.01 ppm (t, J = 1.6 Hz, 1 H; H−C(2′)). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 20.64 (CHMe_2), 36.23 (CHMe$_2$), 52.66 (OMe), 88.95 (C(CN)$_2$), 110.87 and 111.31 (C(CN)$_2$), 114.75 (C(3′)), 117.06 (NC−C(3′)), 126.08 (C(4)), 128.46 (C(6′)), 129.57 (C(4′)), 131.11 (C(2′)), 134.58 (C(3)), 135.03 (C(2)), 135.99 and 136.11 (C(1′,5′)), 146.08 (C(5)), 162.09 (CO_2), 182.71 (C=C(CN)$_2$) ppm. IR (ATR): $\tilde{\nu}$ = 3099 (w), 2980 (w), 2234 (w), 1708 (s), 1699 (s), 1591 (w), 1570 (w), 1537 (w), 1467 (m), 1428 (m), 1418 (m), 1366 (w), 1345 (w), 1313 (m), 1277 (s), 1245 (m), 1225 (m), 1185 (w), 1162 (w), 1096 (s), 1005 (w), 992 (w), 955 (w), 918 (w), 887 (s), 828 (m), 790 (m), 744 (s), 724 (m), 689 (m), 653 (w), 626 (m), 613 cm^{-1} (w). HR-MALDI-MS (negative mode) m/z (%): 360.0812 (100, $[M - H]^-$; calcd for $C_{20}H_{14}N_3O_2S^-$, 360.0812).

Benzyl 4-(3-Cyano-5-isobutyrylphenyl)piperazine-1-carboxylate (33). Under N_2, a mixture of 3-bromo-5-isobutyrylbenzonitrile **12** (756 mg, 3 mmol), benzyl piperazine-1-carboxylate (0.64 mL, 3.3 mmol), NaO^{t}Bu (317 mg, 3.3 mmol), $[Pd_2(dba)_3]$ (92 mg, 0.1 mmol), and 2-biphenyl-di-*tert*-butylphosphine (90 mg, 0.3 mmol) in toluene (5 mL) was stirred at 45 °C for 48 h in a sealed tube. The mixture was filtered through a pad of Celite and evaporated. Column chromatography: (SiO_2; n-hexane/EtOAc 4:1 → 3:1) gave **33** (768 mg, 65%) as an off-white powder. R_f = 0.34 (SiO_2; n-hexane/EtOAc 4:1); mp 127−128 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.21 (d, J = 6.8 Hz, 6 H; CHMe_2), 3.26 (br t, $J \approx$ 5.0 Hz, 4 H; $(CH_2)_2$N−Ar), 3.46 (heptet, J = 6.9 Hz, 1 H; CHMe$_2$), 3.68 (br t, J = 5.2 Hz, 4 H; $(CH_2)_2$N−CO), 5.17 (s, 2 H; CH_2Ph), 7.26 (br dd, J = 2.6, 1.3 Hz, 1 H, H−C(2)), 7.30−7.41 (m, 5 H; Ph), 7.62 (br t, J = 1.3 Hz, 1 H; H−C(4)), 7.66 ppm (br dd, J = 2.7, 1.4 Hz, 1 H; H−C(6)). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 19.12 (CHMe_2), 35.87 (CHMe$_2$), 43.47 (br, $(CH_2)_2$N−CO), 48.30 ($(CH_2)_2$N−Ar), 67.60 (CH_2Ph), 113.81 (C(3)), 118.59 (CN), 119.41 (C(6)), 122.29 (C(2)), 122.57 (C(4)), 128.17 (C(2,6) of Ph), 128.35 (C(4) of Ph), 128.71 (C(3,5) of Ph), 136.53 (C(1) of Ph), 138.09 (C(5)), 151.65 (C(1)), 155.24 (CO_2N), 202.84 ppm (C=O). IR (ATR): $\tilde{\nu}$ = 2966 (w), 2934 (w), 2229 (w), 1682 (s), 1592 (m), 1428 (s), 1361 (m), 1288 (m), 1241 (m), 1214 (s), 1111 (m), 1079 (m), 1032 (m), 1013 (m), 996 (m), 977 (m), 911 (w), 869 (w), 752 (s), 721 (m), 697 (m), 680 (m), 632 cm^{-1} (m). HR-MALDI-MS m/z (%): 392.1969 (100, $[M + H]^+$; calcd for $C_{23}H_{26}N_3O_3^+$, 392.1974). Elemental analysis (%) calcd for $C_{23}H_{25}N_3O_3$ (391.47): C 70.57, H 6.44, N 10.73. Found: C 70.50, H 6.45, N 10.60.

Benzyl 4-[3-Cyano-5-(1,1-dicyano-3-methylbut-1-en-2-yl)-phenyl]piperazine-1-carboxylate (34). Under N_2, a solution of **33** (666 mg, 1.7 mmol) and malononitrile (449 mg, 6.8 mmol) in $CHCl_3$ (7 mL) was treated with a 1 M solution of $TiCl_4$ in toluene (1.7 mL, 1.7 mmol). Pyridine (0.27 mL, 3.4 mmol) was added, and the mixture was stirred at 75 °C for 48 h. 1 M aqueous HCl (100 mL) and EtOAc (100 mL) were added. The solid precipitate was filtered off, and the layers were separated. The aqueous phase was extracted with EtOAc (3 × 50 mL). The combined organic phases were dried over Na_2SO_4 and evaporated. Column chromatography (SiO_2; n-hexane/EtOAc 4:1) gave **34** (390 mg, 52%) as an orange resin. R_f = 0.19 (SiO_2; n-hexane/EtOAc 4:1). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.18 (d, J = 6.9 Hz, 6 H; CHMe_2), 3.27 (br t, J = 5.1 Hz, 4 H; $(CH_2)_2$N−Ar), 3.43 (heptet, J = 6.9 Hz, 1 H; CHMe$_2$), 3.68 (dd, J = 4.1, 6.4 Hz, 4 H; $(CH_2)_2$N−CO), 5.16 (s, 2 H; CH_2Ph), 6.85 (dd, J = 1.5, 2.5 Hz; H−C(6)), 6.89 (t, J = 1.3 Hz, 1 H; H−C(4)), 7.22 (dd, J = 1.3, 2.5 Hz, 1 H; H−C(2)), 7.25−7.60 ppm (m, 5 H; Ph). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 20.36 (CHMe_2), 35.98 (CHMe$_2$), 43.01 (br, $(CH_2)_2$N−CO), 47.68 ($(CH_2)_2$N−Ar), 67.24 (CH_2Ph), 87.79 (C(CN)$_2$), 111.06 and 111.48 (C(CN)$_2$), 113.95 (C(3)), 117.82 and 117.91 (NC−C(3), C(6)), 119.76 and 119.82 (C(2,4)), 127.81 (C(2,6) of Ph), 128.03 (C(4) of Ph), 128.42 (C(3,5) of Ph), 135.84 (C(5)), 136.28 (C(1) of Ph), 150.96 (C(1)), 154.88 (CO_2N), 184.15 ppm (C=C(CN)$_2$). IR (ATR): $\tilde{\nu}$ = 2974 (w), 2232 (m), 1694 (s), 1586 (s), 1497 (w), 1427 (s), 1387 (m), 1360 (m), 1288 (m), 1237 (s), 1223 (s), 1162 (m), 1121 (s), 1078 (m), 1018 (m), 993 (s), 912 (w), 866 (m), 844 (m), 749 (s), 695 (s), 666 cm^{-1} (m). HR-MALDI-MS m/z (%): 462.1901 (100, $[M + Na]^+$; calcd for $C_{26}H_{25}N_5NaO_2^+$, 462.1900).

■ ASSOCIATED CONTENT

Supporting Information

Additional figures on ligand interactions, detailed biological activity, synthetic procedures, compound characterization, and NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

M.C.W., M.R., F.S., P.Chi., P.Cha., and F.D. designed the research; A.S., U.L., P.Chi., M.S., S.T., G.S., C.M., F.T., C.F., A.J., C.P., P.R., M.O., L.M.S.-A., S.C., S.W., and Y.Y. performed research; M.C.W., M.R., U.L., P.Chi., T.M., A.J., M.H., P.M., P.Cha., and F.D. analyzed data; M.C.W., M.R., A.S., G.S., P.Cha., and F.D. wrote the paper.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

CDI, carbonyldiimidazole; DHF, dihydrofolate; DHFR, dihydrofolate reductase; DHFS, dihydrofolate synthase; DHP, dihydropteroate; DHPS, dihydropteroate synthase; dTMP, deoxythymidine monophosphate; dUMP, deoxyuridine monophosphate; *p*ABA, para-aminobenzoic acid; *Pb*, *Plasmodium berghei*; *Pf*, *Plasmodium falciparum*; PLP, pyridoxal 5′-phosphate; *Pv*, *Plasmodium vivax*; SHMT, serine hydroxymethyltransferase; THF, tetrahydrofolate; TS, thymidylate synthase; 5,10-CH_2-THF, 5,10-methylenetetrahydrofolate

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