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Synthesis and Biological Activity of Pyrido[3′,2′:4,5]thieno[3,2-*d*]pyrimidines as Phosphodiesterase Type 4 Inhibitors

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A series of pyrido[3′,2′:4,5]thieno[3,2-*d*]pyrimidines (PTP) has been synthesized and tested as phosphodiesterase IV inhibitors (PDE4), a target for the treatment of asthma and chronic obstructive pulmonary disease (COPD). Structure−activity relationships within this series, leading to an increase of potency on the enzyme, are presented. The *gem*-dimethylcycloalkyl moiety fused to the pyridine ring proved to be a key element of the scaffold in order to get a higher affinity in the enzyme.

Introduction

The guidelines for the treatment of asthma and chronic obstructive pulmonary disease (COPD[a]) show similarities in terms of treatment options available, including the use of inhaled corticosteroids alone or in combination with beta-2 adrenergic agonists and drugs with anticholinergic activity.[1,2]

However, the use of inhaled corticosteroids in asthma and COPD still have some unmet medical needs. In asthma, the concerns are related to the potential local and systemic dose-related adverse effects, whereas in COPD, there is a need for a nonsteroidal anti-inflammatory agent, because inhaled corticosteroids have limited effectiveness.[3]

In the past years, attention has been primarily focused on cyclic nucleotide phosphodiesterase IV (PDE4) as a suitable target for anti-inflammatory therapy in respiratory diseases.[3,4] The mixed anti-inflammatory and bronchodilatory profile of PDE4 inhibitors could allow the discovery of new agents, steroid−sparing compounds with utility in diseases associated with chronic airway inflammation, particularly in the management of asthma and COPD.

PDE4 isoenzymes (PDE4A−D) are encoded by 4 genes and more than 20 splice variants providing the basis for the continued interest in developing selective PDE4 inhibitors for a number of inflammatory diseases.[5] Selective inhibition of PDE4A and/or PDE4B in pro-inflammatory and immune cells is believed to evoke the therapeutically desired effects of these drugs.[6] On one hand, aiming for PDE4B isoform-selective inhibitors was suggested as a work-around to reduce the major toxicity concerns, that is, emesis and the risk of cardiotoxicity potentially related with PDE4D isoform inhibition.[7,8]

Chemically diverse classes of molecules have been reported as PDE4 inhibitors, rolipram-related compounds, xanthine derivatives, or nitraquazone analogues, among others.[4] We report herein some derivatives of pyrido[3′,2′:4,5]thieno[3,2-*d*]-pyrimidines (PTP) with different substituents starting from a lead compound which was identified in an HTS exercise. The optimization process was driven by a typical SAR method.

PDE4 activity from various human recombinant PDE4 subtypes (PDE4B1, PDE4A4, and PDE4D3) was monitored in order to see whether selectivity was achieved. The compounds were also tested to see if they were capable of blocking the production of some pro-inflammatory cytokines such as TNFα.

Chemistry

Here, we describe the synthesis of a variety of new PTP derivatives, which were prepared with the objective of studying the potential of this scaffold to deliver the desired activity profile. These compounds were synthesized as outlined in Schemes 1 and 2.

Ketone **1** is condensed with malononitrile in the presence of carbon disulfide to yield heterocycle **2**.[9] Ketones **1** are commercially available or prepared according to already known methods.[10]

Reaction of compound **2** with a secondary amine, like morpholine, yields the pyridine derivative **3**.[11] Subsequent cyclocondensation of compound **3** with 2-chloroacetamide in the presence of a base such as potassium carbonate affords the thienopyridine compound **4**, according to the procedure described by C. Peinador et al.[12] The pyridothienopyrimidinone derivative **5** is synthesized by cyclization of intermediate **4** with triethylorthoformate. The corresponding chloropyrimidine derivative from **5** is synthesized using phosphorus oxychloride as solvent, and the resulting intermediate is reacted with morpholinoethanamine to give the desired final compound **13**.

This synthetic route is used to obtain products with amine substituents at position 5 of the PTP scaffold. For alkyl or aryl substituents at this position, an alternative route is depicted in Scheme 2.

Regarding Scheme 2, ketone **6** reacts with dimethyl carbonate in the presence of a strong base such as sodium hydride in tetrahydrofurane to yield the diketone **7**.[13] Starting ketones are commercially available or prepared according to already known methods.[10]

Reaction of compound **7** with cyanoacetamide in methanol under refluxing conditions in the presence of potassium

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[a] Abbreviations: PTP, pyrido[3′,2′:4,5]thieno[3,2-*d*]pyrimidines; COPD, chronic obstructive pulmonary disease; PDE4, phosphodiesterase IV.

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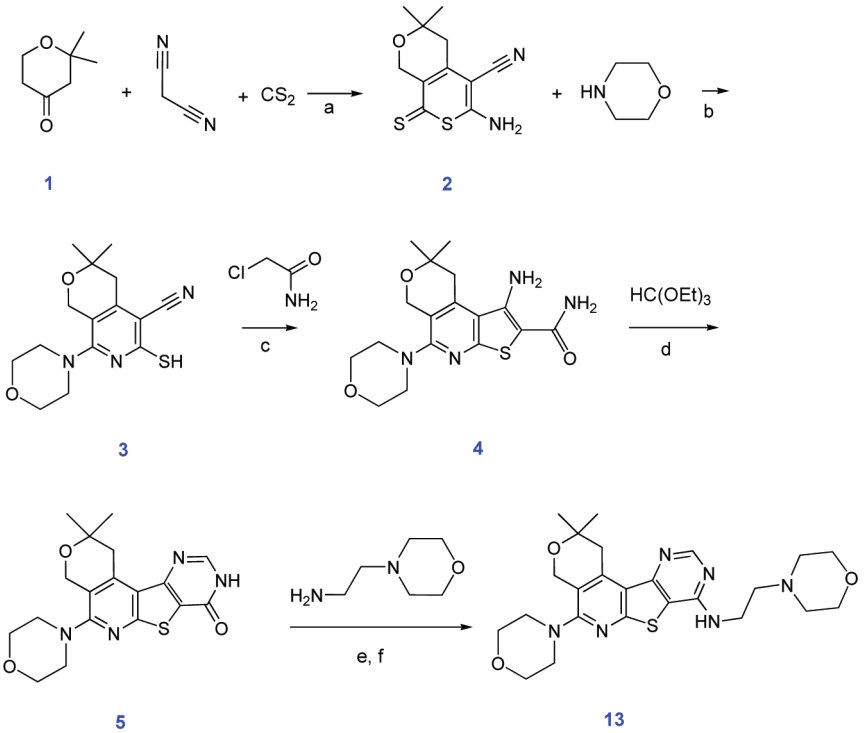
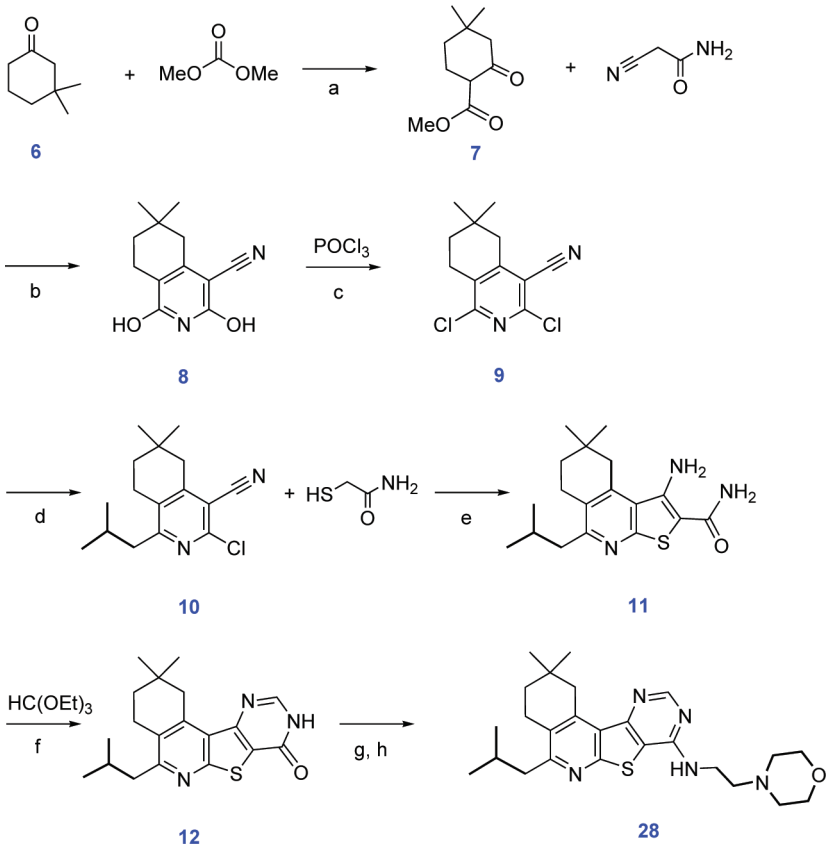
Scheme 1. Synthesis of Pyrido[3′,2′:4,5]thieno[3,2-*d*]pyrimidines[a]



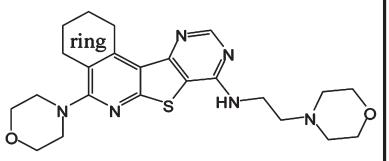
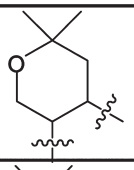
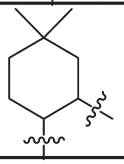
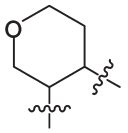
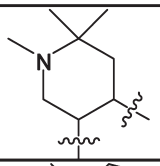
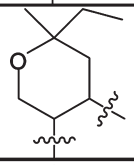
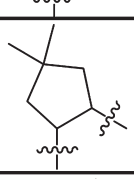
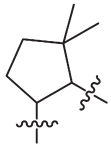
[a] Reagents and conditions: (a) MeOH, TEA, r.t.; (b) EtOH, reflux; (c) K_2CO_3, acetone, reflux; (d) reflux; (e) $POCl_3$, reflux; (f) EtOH, reflux.

Scheme 2. Alternative Synthesis of Pyrido[3′,2′:4,5]thieno[3,2-*d*]pyrimidines[a]



[a] Reagents and conditions: (a) NaH, THF, reflux; (b) KOH, MeOH, reflux; (c) $POCl_3$, 170 °C; (d) K_2CO_3, $Pd(PPh_3)_4$, dioxane, reflux; (e) K_2CO_3, EtOH, reflux; (f) reflux; (g) $POCl_3$, reflux; (h) EtOH, reflux.

Table 1. SAR on the Pyridine Fused Ring and Selectivity Profile

	Cpnd. nr.	PDE4B1 (nM)	PDE4A4 (nM)	PDE4D3 (nM)	HWB TNF-α (nM)
	**13**	26	63	604	235
	**14**	2,9	74	4,6	1368
	**15**	1765	n.d.	n.d.	n.d.
	**16**	3697	n.d.	n.d.	3272
	**17**	90	n.d.	n.d.	2044
	**18**	333	n.d.	n.d.	n.d.
	**19**	274	n.d.	n.d.	n.d.
Rolipram	stand.	498	241	70	1476

hydroxide yields the pyridine derivative **8**.[14] The same reference applies for the conversion of **8** to the 1,6-dichloropyridine derivative **9** by reaction with phosphorus oxychloride.

9 is converted to **10** under classical Suzuki coupling conditions by reaction with a boronic acid or the corresponding boronate in the presence of potassium carbonate and tetrakis-(triphenylphosphine)palladium(0) under reflux of dioxane, where the boronic acids or their corresponding boronates are commercially available or synthesized by common methodology.

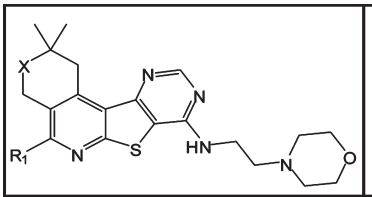
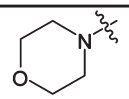
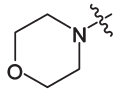
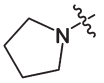
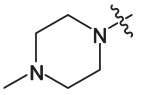
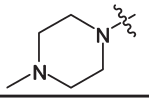
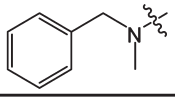
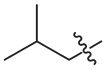
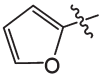
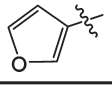
Subsequent cyclocondensation of compound **10** with 2-mercaptoacetamide in the presence of a base such as potassium carbonate affords the thienopyridine compound **11**.[15]

The pyridothienopyrimidinone derivative **12** is synthesized by cyclization of intermediate **11** with triethyl orthoformate.[16] The corresponding chloropyrimidine from **12** is synthesized using phosphorus oxychloride as solvent and reacts with morpholinoethanamine to give the desired final compound **28**.

Results and Discussion

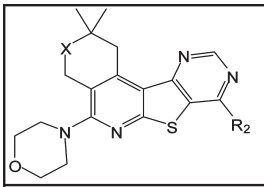
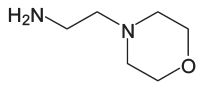
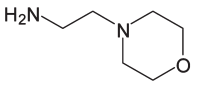
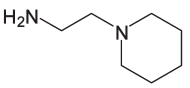
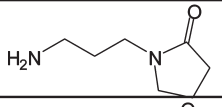
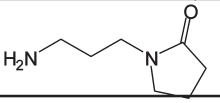
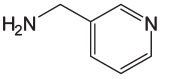
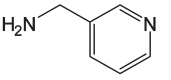
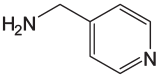
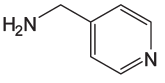
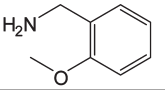
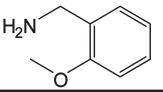
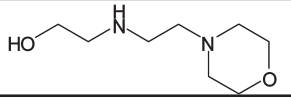
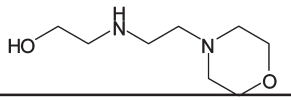
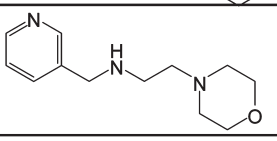
In Table 1, a first group of compounds is presented. They only differ from each other in the ring fused to the PTP central core. According to the PDE4B affinities, it is quite evident that this is a very sensitive part of the

Table 2. SAR at the R_1 Substituent and Selectivity Profile

	Cpnd. nr.	X	PDE4B1 (nM)	PDE4A4 (nM)	PDE4D3 (nM)	HWB TNF-α (nM)
	**13**	O	26	63	604	699
	**14**	C	2,88	74	4,6	1368
	**20**	C	8,05	43	11	655
	**21**	O	1198	n.d.	n.d.	n.d.
	**22**	C	95	n.d.	n.d.	n.d.
	**23**	C	865	n.d.	n.d.	n.d.
MeHN-	**24**	O	51	n.d.	n.d.	n.d.
MeHN-	**25**	C	9,9	18	4,2	233
Me_2N-	**26**	O	45	20	25	261
Me	**27**	C	37	97	34	912
	**28**	C	7,2	6,7	39	1276
	**29**	C	3,8	5,4	7,9	1089
	**30**	C	2,9	9	3,1	1243
Rolipram	stand.	_	498	241	70	1476

inhibitor. It is remarkable that the substitution of the fused tetrahydropyranyl oxygen by a methylene group favors the affinity for the enzyme: **14** (PDE4B1 $IC_{50} = 2.9$ nM) is 1 order of magnitude more potent than **13** (PDE4B1 $IC_{50} =$ 26 nM). The absence of the *gem*-dimethyl substitution at the fused tetrahydropyrane cycle leads to an evident loss of PDE4B1 activity (see **15** compared to **13**), and a similar effect is obtained with a fused cyclopentyl ring instead of the cyclohexyl one, no matter what the position of the *gem*-dimethyl substitution is (**18** and **19** are 2 orders of

Table 3. SAR at the R_2 Substituent and Selectivity Profile

	Cpnd. nr.	X	PDE4B1 (nM)	PDE4A4 (nM)	PDE4D3 (nM)	HWB TNF-α (nM)
	**13**	O	26	63	604	699
	**14**	C	2,9	74	4,6	1368
	**31**	O	284	n.d.	n.d.	n.d.
	**32**	O	167	n.d.	n.d.	n.d.
	**33**	C	21	18	61	2152
	**34**	O	5,7	48	52	639
	**35**	C	3,6	4,5	32	381
	**36**	O	22	97	128	1022
	**37**	C	11	19	13	2044
	**38**	O	40	67	119	1191
	**39**	C	213	n.d.	n.d.	n.d.
	**40**	O	13	23	39	537
	**41**	C	2,8	6,4	1,8	327
	**42**	O	4,5	5	19	401
Rolipram	stand.	_	498	241	70	1476

magnitude less potent than **14**). Moreover, this *gem*-dimethyl substitution seems to be the optimum pattern to fill the putative lipophilic pocket at the PDE4 active site: if one of the methyls is substituted by an ethyl, the PDE4B1 inhibitor activity drops from 26 nM (**13**) to 90 nM (**17**, racemic mixture). This loss is much more pronounced in the

case where the oxygen of the tetrahydropyrane ring is replaced by an *N*-methyl moiety, leading to the inactive molecule **16**. For our study, both **13** and **14** will be our reference compounds.

The general low potency showed at inhibiting the PDE4B1 enzyme restrained us to test these compounds in other PDE4 subtypes, except for products **13** and **14**. In the particular case of **13**, a selectivity of 1 order of magnitude was achieved as is observed by comparing the PDE4B1 data (26 nM) with that of PDE4D3 (604 nM); nevertheless, the HWB TNF-α value (235 nM) is weak compared to the enzymatic values, which can be explained through the high plasma protein binding value (92.17% for **13**). In the case of **14**, its low Caco-2 value (Papp A−B = 3.1 (cm/s)× 10^{-6}) gave us another clue to explain such a residual HWB TNF-α activity (1368 nM).

Concerning the compounds in Table 2, they all have the *gem*-dimethyl substitution at the fused cycle (either cyclohexyl or tetrahydropyran) and the 2-morpholin-4-ylethyl moiety at the 8-nitrogen, but differ in the substituent of the 5-position of the PTP central core. In the cyclohexyl series (X = C), pyrrolidine (**20**) and methylamino (**25**) were rather equivalent to the morpholine (**14**). The same tendency is observed in the pyranyl series (X = O), although the analogues in this case tend to be less potent (compare **24** or **26** and **25**). At this position, groups like *N*-methylpirazine (**21**) or *N*-benzylmethylamine (**23**) are not welcome, potency at the enzyme dropping almost 2 orders of magnitude in the tetrahydropyran series, although naked furyl ring retains initial potency (compare **29** and **30** to **14**). Finally, in the cyclohexyl series, alkyl groups like methyl (**27**) or isobutyl (**28**) are also a good choice, the latter being a bit more potent. In conclusion, it seems that small lypophilic groups give good potencies, whereas the bigger ones are susceptible to be charged and give poorer activities.

It is noteworthy to observe that in this set of compounds no selectivity among PDE4 subtypes is achieved compared to product **13** data, with **28** perhaps the only exception (39 nM in PDE4D3 in front of 7.21 nM in PDE4B1). Besides, acceptable Caco-2 values for compounds **25** (Papp A−B = 13.7 (cm/s) × 10^{-6}) and **26** (Papp A−B = 21.9 (cm/s) × 10^{-6}) could justify moderately active HWB TNF-α data (233 nM and 261 nM, respectively), which is not the case of compound **28** (Papp A−B = 0.4 (cm/s) × 10^{-6}), a disappointing but expected value taking into account its activity in the HWB TNF-α assay (1276 nM).

According to the results shown in Table 3, substitution at position 8 was not a very sensitive part of the scaffold, except for piperidinethylamino (**31**), which loses ten times in potency with relation to **13**. The discovered tendency of the cyclohexyl series to be more potent than the pyran one (compare **13** with **14**) is no longer the case when substituting this 8-nitrogen by pyridine-3-ylmethyl or pyridine-4ylmethyl groups (see compounds from **34** to **37**). Even a reversed example (2-methoxybenzyl) is reported (compare **38** with **39**), although that pattern is followed in the case of *N*-propylpyrrolidone (compare **32** with **33**) and 2-(2-morpholinoethylamino)ethanol (compare **40** with **41**). This part of the inhibitor may be located in a relatively uncrowded area of the active site, thus explaining the astonishing result of **42** (4.5 nM), one of the most potent compounds even with such a bulky residue. The variability of substituents accepted at this position (small and large residues, charged and noncharged moieties, lipophilic and hydrophilic groups) makes it ideal for potential modulation of the compound's physicochemical properties.

Besides entry **13**, in this last set of compounds a couple of examples can be identified as selective molecules, namely, **34** and **35**, both an order of magnitude less potent in PDE4D3 than in PDE4B1. As we have seen in previous examples, weak values at HWB TNF-α could be explained by a high plasma protein binding result: 98.35% for **34**; 100% for **35**. On the other hand, less weak HWB TNF-α values for compounds **40** (537 nM) and **42** (401 nM) are in good correlation with their Caco-2 data (Papp A−B = 38.7 (cm/s) × 10^{-6} for **40** and 24.3 (cm/s) × 10^{-6} for **42**).

Conclusion

From biological data and SAR analysis using a diverse set of substituents, the pyrido[3′,2′:4,5]thieno[3,2-*d*]pyrimidine scaffold has shown potential to produce new PDE4 inhibitors. The biological data reported in Tables 1 and 2 confirm the importance of the *gem*-dimethylcyclohexyl group, as well as the substitution at position 5, whereas the substitution at the position 8 (see Table 3) has less influence in achieving significant enzymatic activity. Further investigation is on course in order to complete the profile of a PDE4 inhibitor candidate for development.

Experimental Section

Chemistry: General. Reagents, starting materials, and solvents were purchased from commercial suppliers and used as received. 2,2-Dimethyltetrahydropyran-4-one (**1**) was provided by Matrix Scientific (cat. no. 021441) or by Aldrich (cat. no. 198242); 3,3-dimethylcyclohexanone (**6**) was purchased from Aldrich (cat.no. 674893); 3,3-dimethylcyclopentanone was synthesized according to Paquette et al.;[16] methyl-4,4-dimethyl-2-oxo-cyclohexanecarboxylate (**7**) was synthesized as described in the literature;[13] 1,3-dihydroxy-6,6-dimethyl-5,6,7,8-tetrahydroisoquinoline-4-carbonitrile (**8**) and 1,3-dichloro-6,6-dimethyl-5,6,7,8-tetrahydroisoquinoline-4-carbonitrile (**9**) were prepared according to the method described by Wenkert;[14] 2,2-dimethylcyclopentanone was commercially available at Aldrich (cat. no. 31,147−5); 2,2,3-trimethyl-5-morpholin-4-yl-1,2,3,4-tetrahydropyrimido[4′,5′:4,5]-thieno[2,3-*c*]-2,7-naphthyridin-8(9*H*)-one and 2-ethyl-2-methyl-5-morpholin-4-yl-1,4-dihydro-2*H*-pyrano[4″,3″:4′,5′]pyrido[3′,2′:4,5]thieno[3,2-*d*]pyrimidin-8(9*H*)-one were commercially available. Concentration refers to evaporation under vacuum using a Büchi rotatory evaporator. Reaction products were purified, when necessary, by flash chromatography on silica gel (40−63 μm) with the solvent system indicated. ^{1}H NMR spectra were performed in a Varian Gemini 2000 spectrometer operating at a frequency of 200 or 300 MHz. Samples were solved in deuterated chloroform ($CDCl_3$) or deuterated dimethylsulfoxide (DMSO-d_6). Tetramethylsilane (TMS) was used as reference. The following abbreviations were used to assign spectra: s, singlet; d, doublet; dd, double doublet; ddd, double double doublet; t, triplet; dt, double triplet; td, triple doublet; m, multiplet; br.s, broad signal. HPLC-UV-MS chromatograms were acquired in a Waters Alliance 2695 chromatographer equipped with a Waters 2996 diode-array detector and a Waters ZQ mass spectrometer detector. HPLC analysis was conducted according to the described method, with the retention time (t_R) expressed in min. UV chromatograms were processed at 210 nm with blank subtraction. HPLC method: Chromatography performed on a Symmetry C18 column (100 × 2.16 mm, 3.5 μm). The mobile phase, at a flow of 0.4 mL/min, was a 20 min binary gradient of water (containing 0.01 M ammonic formate at pH 3.0) and a mixture of acetonitrile−methanol 50:50 (containing 0.01 M ammonic formate) (0−95%). The total run time was 26 min.

6-Amino-3,3-dimethyl-8-thioxo-4,8-dihydro-1*H*-3*H*-thiopyrano-[3,4-*c*]pyran-5-carbonitrile (2). 5.0 g (32.0 mmol) of compound **1** was dissolved in 4.7 mL of methanol, and 4.7 mL (48.8 mmol) of

carbon disulfide was added in one portion. 2.6 g (39.0 mmol) of malonodinitrile was added portionwise followed by a final addition of 1.95 mL of triethylamine. The reaction mixture was stirred at room temperature for 48 h. An orange precipitate formed, which was filtered and washed with diethyl ether. 3.90 g of the final product was isolated. From the filtrates, an additional 0.89 g was obtained after purification by flash chromatography, eluting first with CH_2Cl_2 and then with a mixture of CH_2Cl_2/MeOH (98:2). Yield = 48%. ^{1}H NMR (200 MHz, $CDCl_3$) δ ppm 1.30 (s, 6 H), 2.62 (s, 2 H), 4.66 (s, 2 H), 7.91 (s, 2 H).

General Procedure for Mercaptoaminopyridinecarbonitriles 3. 6-Mercapto-3,3-dimethyl-8-morpholin-4-yl-3,4-dihydro-1*H*-pyrano[3,4-*c*]pyridine-5-carbonitrile (3). 3.9 g (15.45 mmol) of compound **2** was suspended in 17 mL of ethanol, and 6.7 mL (77.3 mmol) of morpholine was added to the resulting suspension. The reaction mixture was refluxed under nitrogen overnight. Then, it was allowed to reach room temperature, and the reaction mixture was left in an ice bath for two hours. The solid formed was filtered and washed twice with ethanol. After drying, 3.12 g of the title compound was obtained as a dark solid, pure enough to perform the next step. Yield = 66%. ^{1}H NMR (200 MHz, $CDCl_3$) δ ppm 1.30 (s, 6 H), 2.75 (s, 2 H), 3.3 (m, 4 H), 3.75 (m, 4H), 4.5 (s, 2H).

General Procedure for Thienopyridines 4 and 11. 1-Amino-8,8-dimethyl-5-morpholin-4-yl-8,9-dihydro-6*H*-pyrano[4,3-*d*]thieno-[2,3-*b*]pyridine-2-carboxamide (4). To a suspension of 6-mercapto-3,3-dimethyl-8-morpholin-4-yl-3,4-dihydro-1*H*-pyrano[3,4-*c*]-pyridine-5-carbonitrile **3** (3.12 g, 10.22 mmol) in ethanol (150 mL), potassium carbonate (3.3 g, 24.5 mmol) and 2-chloroacetamide (1.05 g, 11.24 mmol) were added, and the reaction mixture was then refluxed for 4 h under nitrogen. The solvent was evaporated under vacuum and water was then added to the residue. The precipitated solid was filtered and dried, yielding 3.0 g (81%) of the title compound. ^{1}H NMR (200 MHz, DMSO-d_6) δ ppm 1.29 (s, 6 H), 3.08 (m, 4 H), 3.20 (s, 2 H), 3.73 (m, 4 H), 4.64 (s, 2 H), 6.81 (s, 2 H), 7.07 (s, 2 H).

3-Chloro-1-isobutyl-6,6-dimethyl-5,6,7,8-tetrahydroisoquinoline-4-carbonitrile (10). 1,3-Dichloro-6,6-dimethyl-5,6,7,8-tetrahydroisoquinoline-4-carbonitrile **9** (0.5 g 1.96 mmol) reacted with isobutylboronic acid (0.24 g, 2.35 mmol), potassium carbonate (0.81 g, 5.88 mmol), and tetrakistriphenylphosphine palladium (0.27 g, 0.19 mmol) by refluxing overnight in dioxane (10 mL). Once the reaction was over, the solvent was evaporated under reduced pressure and the residue worked up as usual with dichloromethane and water. After flash chromatography eluting with CH_2Cl_2/MeOH 7:3, 0.26 g of the final compound is isolated. Yield = 47%. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 0.95 (d, 6H), 1.10 (s, 6 H), 1.65 (t, 2 H), 2.20 (dq, 1H), 2.65 (d, 2H), 2.75 (m, 4 H). GC: 95%, t_R = 8.1 min, MS(ESI) 276 m/z (M^+).

1-Amino-5-isobutyl-8,8-dimethyl-6,7,8,9-tetrahydrothieno[2,3-*c*]-isoquinoline-2-carboxamide (11). 3-Chloro-1-isobutyl-6,6-dimethyl-5,6,7,8-tetrahydroisoquinoline-4-carbonitrile (10) (0.26 g, 0.92 mmol) is dissolved in ethanol (5 mL). To this solution, potassium carbonate (0.31 g, 2.22 mmol) and thioacetamide (1.01 mL, 1.11 mmol) are added and the mixture is heated overnight to 100 °C. After the usual workup, 0.31 g (yield = 99%) of the desired final compound is obtained as a yellow solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 0.95 (d, 6H), 1.10 (s, 6 H), 1.65 (t, 2 H), 2.20 (dq, 1H), 2.65 (d, 2H), 2.80 (t, 2 H), 3.05 (s, 2H), 5.30 (br.s, 2H), 6.50 (br.s, 2H).

General Procedure for Thienopyridines 5 and 12. 2,2-Dimethyl-5-morpholin-4-yl-1,4-dihydro-2*H*-pyrano[4″,3″:4′,5′]pyrido[3′,2′:4,5]thieno[3,2-*d*]pyrimidin-8(9*H*)-one (5). 3.0 g (8.3 mmol) of **4** was suspended in triethyl orthoformate (50 mL) and *p*-toluensulfonic acid hydrate (0.16 g, 0.83 mmol) was added. This mixture was heated under reflux overnight. The reaction mixture was then allowed to reach room temperature and it was left in an ice bath for two hours. The precipitated that formed was filtered and washed with ethyl ether. After drying, 2.8 g of the final product was obtained. Yield = 92%. ^{1}H NMR (200 MHz, DMSO-d_6) δ ppm 1.32 (s, 6 H), 3.20 (m, 4 H), 3.44 (s, 2 H), 3.76 (m, 4 H), 4.70 (s, 2 H), 8.33 (s, 1 H).

2,2-Dimethyl-5-isobutyl-1,2,3,4-tetrahydropyrimido[4′,5′:4,5]-thieno[2,3-*c*]isoquinolin-8(9*H*)-one (12). Prepared following the general method described for the synthesis of compound **5** but starting from 0.31 g (0.92 mmol) of **11**. Reaction time: 3 h. 0.23 g of the final product was obtained. Yield = 72%. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 0.95 (d, 6H), 1.10 (s, 6 H), 1.70 (t, 2 H), 2.30 (dq, 1H), 2.80 (d, 2H), 2.90 (t, 2 H), 3.40 (s, 2H), 8.30 (s, 1H). MS (ESI) 341 m/z (M^+).

General Procedure for Thienopyridines 13 and 28. 2.84 g (7.63 mmol) of **5** was suspended in phosphorus oxychloride (30 mL) and heated to reflux for 90 min. The excess phosphorus oxychloride was evaporated under vacuum and the residue partitioned between chloroform and a cooled 2 N aqueous solution of NaOH. The aqueous phase was extracted twice with chloroform, and the organic phases were washed with water and brine, dried over magnesium sulfate, filtered, and the solvent evaporated. 2.98 g of 8-chloro-2,2-dimethyl-5-morpholin-4-yl-1,2,3,4-tetrahydropyrimido[4′,5′:4,5]thieno[2,3-*c*]isoquinoline was obtained. Yield = 100%. ^{1}H NMR (200 MHz, $CDCl_3$) δ ppm 1.44 (s, 6 H), 3.35 (m, 4 H), 3.57 (s, 2 H), 3.88 (m, 4 H), 4.78 (s, 2 H), 9.02 (s, 1 H).

8-Chloro-2,2-dimethyl-5-morpholin-4-yl-1,2,3,4-tetrahydropyrimido[4′,5′:4,5]thieno[2,3-*c*]isoquinoline (1.00 g, 2.56 mmol) is suspended in ethanol (60 mL) and (2-morpholin-4-ylethyl)-amine (1.68 mL, 12.8 mmol) is added. The mixture is refluxed overnight and then allowed to cool to room temperature. At +5 °C, a precipitate is formed, which is filtered and washed with ethanol and ethyl ether. Once dried, it weighs 0.70 g and its ^{1}H NMR is consistent with the desired final compound **13**. Yield = 56%. ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 1.32 (s, 6 H), 2.46 (d, J = 4.27 Hz, 3 H), 2.48−2.53 (m, 3 H), 2.56 (t, J = 6.87 Hz, 2 H), 3.11−3.24 (m, 4 H), 3.35 (s, 2 H), 3.54−3.60 (m, 4 H), 3.71−3.85 (m, 4 H), 4.70 (s, 2 H), 7.71 (t, J = 5.65 Hz, 1 H), 8.58 (s, 1 H). HPLC: 99.0%, t_R = 9 min, MS(ESI) 485 m/z $(M+1)^+$.

2,2-Dimethyl-5-morpholin-4-yl-*N*-(2-morpholin-4-ylethyl)-1,2,3,4-tetrahydropyrimido[4′,5′:4,5]thieno[2,3-*c*]isoquinolin-8-amine (14). Obtained (62%) from compound **6** and (2-morpholin-4-ylethyl)amine following the experimental procedure described for compound **13**. mp 210.2−210.9 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 1.07 (s, 6 H), 1.55 (t, J = 6.10 Hz, 2 H), 1.55 (t, J = 6.10 Hz, 2 H), 2.36 (s, 1 H), 2.45 (m, 2 H), 2.56 (t, J = 6.87 Hz, 2 H), 2.75 (t, J = 6.10 Hz, 2 H), 3.20 (m, 4 H), 3.43 (m, 1 H), 3.57 (m, 2 H), 3.63 (m, 2 H), 3.78 (m, 4 H), 7.64 (t, J = 5.49 Hz, 1 H), 8.59 (s, 1 H). HPLC: 93.20%, t_R = 12 min, MS(ESI) 483 m/z $(M+1)^+$.

5-Morpholin-4-yl-*N*-(2-morpholin-4-ylethyl)-1,4-dihydro-2*H*-pyrano[4″,3″:4′,5′]pyrido[3′,2′:4,5]thieno[3,2-*d*]pyrimidin-8-amine (15). Obtained (43%) from tetrahydropyran-4-one and (2-morpholin-4-ylethyl)amine following the experimental procedure described for compound **13**. mp 184.5−185.3 °C. ^{1}H NMR (300 MHz, CD_3OD) δ ppm 2.69 (t, J = 6.87 Hz, 2 H), 3.22 (m, 4 H), 3.76 (m, 16 H), 4.10 (t, J = 6.10 Hz, 2 H), 4.79 (m, 2 H), 8.52 (s, 1 H). HPLC: 93.6%, t_R = 9 min, MS(ESI) 457 m/z $(M+1)^+$.

2,2,3-Trimethyl-5-morpholin-4-yl-*N*-(2-morpholin-4-ylethyl)-1,2,3,4-tetrahydropyrimido[4′,5′:4,5]thieno[2,3-*c*]-2,7-naphthyridin-8-amine (16). Obtained (53%) from commercially available 8-oxo-2,2,3-trimethyl-5-morpholin-4-yl-1,2,3,4-tetrahydropyrimido[4′,5′:4,5]thieno[2,3-*c*]-2,7-naphthyridine and (2-morpholin-4-ylethyl)amine following the experimental procedure described for compound **13**. mp 103.2−105.1 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 1.13 (s, 6 H), 2.31 (m, 2 H), 2.50 (m, 4 H), 2.57 (m, 1 H), 3.20 (s, 4 H), 3.33 (d, J = 7.02 Hz, 5 H), 3.57 (m, 4 H), 3.63 (s, 4 H), 3.77 (d, J = 5.19 Hz, 4 H), 8.59 (s, 1 H). HPLC: 95.1%, t_R = 6 min, MS(ESI) 498 m/z $(M+1)^+$.

2-Ethyl-2-methyl-5-morpholin-4-yl-*N*-(2-morpholin-4-ylethyl)-1,4-dihydro-2*H*-pyrano[4″,3″:4′,5′]pyrido[3′,2′:4,5]thieno[3,2-*d*]-pyrimidin-8-amine (17). Obtained (49%) from commercially available 8-oxo-2-ethyl-2-methyl-5-morpholin-4-yl-1,4-dihydro-*2H*-pyrano[4″,3″:4″,5′]pyrido[3′,2′:4,5]thieno[3,2-*d*]pyrimidine and

(2-morpholin-4-ylethyl)amine following the experimental procedure described for compound **13**. mp 92.5−93.8 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 0.92 (t, J = 7.48 Hz, 3 H), 2.45 (d, J = 4.27 Hz, 2 H), 2.51 (m, 4 H), 2.56 (t, J = 7.02 Hz, 2 H), 3.18 (m, 4 H), 3.33 (d, J = 7.02 Hz, 3 H), 3.50 (d, J = 3.66 Hz, 2 H), 3.57 (m, 4 H), 3.67 (m, 2 H), 3.76 (s, 4 H), 4.67 (d, J = 7.32 Hz, 2 H), 7.73 (m, 1 H), 8.58 (s, 1 H). HPLC (Method B): 96.3%, t_R = 10 min, MS(ESI) 499 m/z $(M+1)^+$.

2,2-Dimethyl-4-morpholin-4-yl-*N*-(2-morpholin-4-ylethyl)-2,3-dihydro-*1H*-cyclopenta[4′,5′]pyrido[3′,2′:4,5]thieno[3,2-*d*]pyrimidin-7-amine (18). Obtained (39%) from 3,3-dimethylcyclopentanone and (2-morpholin-4-ylethyl)amine following the general experimental procedure described for compound **13**. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 1.2 (m, 6 H), 2.6 (s, 4 H), 2.7 (t, J = 5.9 Hz, 2 H), 2.8 (s, 2 H), 3.4 (s, 2 H), 3.6 (m, 4 H), 3.7 (m, 6 H), 3.9 (m, 4 H), 5.6 (m, 1 H), 8.7 (s, 1 H). HPLC: 98.9%, t_R = 11 min, MS(ESI) 469 m/z $(M+1)^+$.

1,1-Dimethyl-4-morpholin-4-yl-N^7-(2-morpholin-4-ylethyl)-2,3-dihydro-*1H*-cyclopenta[4′,5′]pyrido[3′,2′:4,5]thieno[3,2-*d*]pyrimidin-7-amina (19). Obtained (81%) from 2,2-dimethylcyclopentanone and (2-morpholin-4-ylethyl)amine following the general experimental procedure described for compound **13**. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 1.68 (s, 3 H), 1.99 (t, J = 7.26 Hz, 2 H), 2.45 (d, J = 4.15 Hz, 4 H), 2.53−2.61 (m, 2 H), 2.98 (t, J = 7.26 Hz, 2 H), 3.31 (s, 3 H), 3.37−3.47 (m, 4 H), 3.54−3.59 (m, 4 H), 3.60−3.68 (m, 2 H), 3.70−3.80 (m, 4 H), 7.55 (t, J = 5.39 Hz, 1 H), 8.56 (s, 1 H). HPLC: 99.1%, t_R = 12 min, MS (ESI) 469 m/z $(M+1)^+$.

2,2-Dimethyl-*N*-(2-morpholin-4-ylethyl)-5-pyrrolidin-1-yl-1,2,3,4-tetrahydropyrimido[4′,5′:4,5]thieno[2,3-*c*]isoquinolin-8-amine (20). Obtained (50%) from compound **6** and (2-morpholin-4-ylethyl)amine following the experimental procedure described for compound **13**. mp 173.3−174.0 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 1.06 (s, 6 H), 1.50 (t, J = 6.10 Hz, 2 H), 1.90 (m, 4 H), 2.44 (m, 4 H), 2.56 (m, 2 H), 2.77 (t, J = 5.95 Hz, 2 H), 3.30 (s, 2 H), 3.60 (m, 10 H), 7.41 (t, J = 5.49 Hz, 1 H), 8.52 (s, 1 H). HPLC: 98.6%, t_R = 15 min, MS(ESI) 467 m/z $(M+1)^+$.

2,2-Dimethyl-5-(4-methylpiperazin-1-yl)-*N*-(2-morpholin-4-ylethyl)-1,4-dihydro-*2H*-pyrano[4″,3″:4′,5′]pyrido[3′,2′:4,5]thieno-[3,2-*d*]pyrimidin-8-amine (21). Obtained (40%) from compound **5** and *N*-methylpyperazine following the experimental procedure described for compound **13**. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 1.42 (s, 6 H) 2.40, (s, 3 H), 2.54 (br.s, 4 H), 2.64 (br.s, 4 H), 2.72 (s, 2 H), 3.32 (br.s, 4 H), 3.60 (s, 2 H), 3.78 (br.s, 6 H), 4.78 (s, 2 H), 5.61 (br.s, 1 H), 8.71 (s, 1 H). HPLC: 95.6%, t_R = 6 min, MS(ESI) 498 m/z $(M+1)^+$.

2,2-Dimethyl-5-(4-methylpiperazin-1-yl)-*N*-(2-morpholin-4-ylethyl)-1,2,3,4-tetrahydropyrimidino[4′,5′:4,5]thieno[2,3-*c*]isoquinolin-8-amine (22). Obtained (50%) from compound **6** and *N*-methylpiperazine following the experimental procedure described for compound **13**. mp ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 1.13 (s, 6 H), 1.63 (s, 2 H), 2.39 (s, 3 H), 2.55 (br.s, 4 H), 2.62 (br.s, 4 H), 2.71 (s, 2 H), 3.34 (br.s, 4 H), 3.45 (s, 2 H), 3.74 (br.s, 8 H), 5.55 (s, 1 H), 8.72 (s, 1 H). HPLC: 100.0%, t_R = 7 min, MS(ESI) 496 m/z $(M+1)^+$.

N^5-Benzyl-N^5,2,2-Trimethyl-*N*-(2-morpholin-4-ylethyl)-1,2,3,4-tetrahydropyrimido[4′,5′:4,5]thieno[2,3-*c*]isoquinolin-8-amine (23). Obtained (67%) from compound **6** and benzylmethylamine following the experimental procedure described for compound **13**. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 1.12 (s, 6 H), 1.59 (t, J = 6.32 Hz, 4 H), 2.56 (s, 3 H), 2.73 (s, 2 H), 2.84 (t, J = 6.59 Hz, 2 H), 2.88 (s, 4 H), 3.45 (s, 2 H), 3.77 (s, 4 H), 4.49 (s, 2 H), 5.54 (s, 1 H), 7.27−7.48 (m, 5 H), 8.72 (s, 1 H). HPLC: 97.1%, t_R = 18 min, MS(ESI) 517 m/z $(M+1)^+$.

N^5,2,2-trimethyl-N^8-(2-morpholin-4-ylethyl)-1,4-dihydro-*2H*-pyrano[4″,3″:4′,5′]pyrido[3′,2′:4,5]thieno[3,2-*d*]pyrimidin-5,8-diamine (24). Obtained (12%) from compound **5** and methylamine following the experimental procedure described for compound **13**. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 1.38 (s, 6 H), 2.57 (d, J = 4.12 Hz, 4 H), 2.72 (t, J = 5.91 Hz, 2 H), 3.15 (d, J = 4.94 Hz, 3 H), 3.38−3.59 (m, 2 H), 3.64−3.95 (m, 5 H), 4.24 (d, J = 4.94 Hz, 1 H), 4.59 (s, 3 H), 5.55 (br.s, 1 H), 8.67 (s, 1 H). HPLC: 97.9%, t_R = 8 min, MS(ESI) 429 m/z $(M+1)^+$.

N^5,2,2-Trimethyl-N^8-(2-morpholin-4-ylethyl)-1,2,3,4- tetrahydropyrimido[4′,5′:4,5]thieno[2,3-*c*]isoquinolin-5,8-diamine (25). Obtained (51%) from compound **6** and methylamine following the experimental procedure described for compound **13**. mp ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 1.07 (s, 6 H), 1.70 (br.s, 2 H), 2.43 (br.s, 4 H), 2.55 (br.s, 3 H), 2.71 (br.s, 2 H), 3.16 (d, J = 3.30 Hz, 3 H), 3.37 (br.s, 2 H), 3.54−3.90 (m, 5 H), 4.67 (br.s, 1 H), 5.47 (br.s, 1 H), 8.68 (s, 1 H). HPLC: 97.6%, t_R = 11 min, MS (ESI) 427 m/z $(M+1)^+$.

N^5,N^5,2,2-Tetramethyl-N^8-(2-morpholin-4-ylethyl)-1,4-dihydro-*2H*-pyrano[4″,3″:4′,5′]pyrido[3′,2′:4,5] thieno[3,2-*d*]pyrimidine-5,8-diamine (26). Obtained (83%) from compound **1** and (2-morpholin-4-ylethyl)-pyridin-2-ylmethylamine following the experimental procedure described for compound **13**. mp 195.1−195.8 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 1.43 (s, 6 H), 2.55 (s, 4 H), 2.72 (t, J = 6.04 Hz, 2 H), 2.98 (s, 6 H), 3.58 (s, 2 H), 3.74 (m, 6 H), 4.80 (s, 2 H), 5.56 (m, 1 H), 8.70 (s, 1 H). HPLC: 99.8%, t_R = 10 min, MS (ESI) 443 m/z $(M+1)^+$.

2,2,5-Trimethyl-*N*-(2-morpholin-4-ylethyl)-1,2,3,4- tetrahydropyrimido[4′,5′:4,5]thieno[2,3-*c*]isoquinolin-8-amine (27). Obtained (50%) from compound **6** and methylboronic acid following the experimental procedure described for compound **28**. mp ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 1.09 (s, 6 H), 1.63−1.77 (m, 2 H), 2.49−2.58 (m, 4 H), 2.62 (s, 3 H), 2.68−2.74 (m, 2 H), 2.75−2.86 (m, 2 H), 3.45−3.55 (m, 2 H), 3.64−3.87 (m, 6 H), 5.44−5.67 (m, 1 H), 8.68−8.83 (m, 1 H). HPLC (Method B): 95.8%, t_R = 12 min, MS (ESI) 412 m/z $(M+1)^+$.

5-Isobutyl-2,2-dimethyl-*N*-(2-morpholin-4-ylethyl)-1,2,3,4-tetrahydropyrimido[4′,5′:4,5]thieno[2,3-*c*]isoquinolin-8-amine (28). Obtained (68%) from compound **12** and (2-morpholin-4-ylethyl)-amine following the experimental procedure described for compound **13**. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 1.12 (s, 6 H), 1.58 (s, 4 H), 1.87 (t, J = 5.67 Hz, 2 H), 2.07 (quin, J = 7.53 Hz, 2 H), 2.48 (t, J = 8.22 Hz, 3 H), 2.77 (t, J = 6.46 Hz, 2 H), 3.30 (d, J = 4.30 Hz, 4 H), 3.37−3.50 (m, 6 H), 3.66 (q, J = 6.13 Hz, 2 H), 3.88 (d, J = 4.70 Hz, 2 H), 6.29 (t, J = 6.06 Hz, 1 H), 8.69 (s, 1 H). HPLC: 97.7%, t_R = 15 min, MS (ESI) 454 m/z $(M+1)^+$.

5-(2-Furyl)-2,2-dimethyl-*N*-(2-morpholin-4-ylethyl)-1,2,3,4-tetrahydropyrimido[4′,5′:4,5]thieno[2,3-*c*]isoquinolin-8-amine (29). Obtained (75%) from compound **6** and 2-furanylboronic acid following the experimental procedure described for compound **28**. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 1.14 (s, 6 H), 1.57 (s, 10 H), 1.72 (t, J = 6.65 Hz, 1 H), 2.56 (br.s, 2 H), 2.73 (t, J = 6.06 Hz, 2 H), 3.15 (s, 1 H), 3.76 (d, J = 5.09 Hz, 2 H), 5.62 (br.s, 1 H), 6.61 (dd, J = 3.33, 1.76 Hz, 1 H), 7.12 (d, J = 3.52 Hz, 1 H), 7.68 (d, J = 1.96 Hz, 1 H), 8.77 (s, 1 H). HPLC: 95.1%, t_R = 13 min, MS (ESI) 464 m/z $(M+1)^+$.

5-(3-Furyl)-2,2-dimethyl-*N*-(2-morpholin-4-ylethyl)-1,2,3,4-tetrahydropyrimido[4′,5′:4,5]thieno[2,3-*c*]isoquinolin-8-amine (30). Obtained (79%) from compound **6** and 3-furanylboronic acid following the experimental procedure described for compound 28. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 1.13 (s, 6 H) 1.72 (t, J = 6.65 Hz, 2 H) 2.56 (d, J = 4.30 Hz, 2 H) 2.73 (t, J = 6.06 Hz, 2 H) 2.98 (t, J = 6.65 Hz, 2 H) 3.47 (t, J = 7.04 Hz, 1 H) 3.57 (s, 2 H) 3.65 - 3.86 (m, 7 H) 5.60 (br. s., 1 H) 7.12 (d, J = 1.96 Hz, 1 H) 7.54 (d, J = 1.57 Hz, 1 H) 7.97 (s, 1 H) 8.77 (s, 1 H). HPLC: 97.5%, t_R = 13 min, MS (ESI) 464 m/z $(M+1)^+$

2,2-Dimethyl-5-morpholin-4-yl-*N*-(2-piperidin-1-ylethyl)-1,4-dihydro-*2H*-pyrano[4″,3″:4′,5′]pyrido[3′,2′:4,5]thieno[3,2-*d*]pyrimidin-8-amine (31). was obtained (81%) from compound **5** and (piperidin-1-yl-ethyl)amine following the experimental procedure described for compound 13. 1H NMR (300 MHz, DMSO-d_6) δ ppm 1.32 (s, 6 H) 1.37 (br. s., 2 H) 1.47 (br. s., 4 H) 2.41 (br. s., 6 H) 3.19 (br. s., 4 H) 3.50 (s, 2 H) 3.62 (br. s., 2 H) 3.76 (br. s., 4 H) 4.70 (s, 2 H) 7.56 - 7.79 (m, 1 H) 8.58 (s, 1 H). HPLC: 97.3%, t_R = 11 min, MS (ESI) 483 m/z $(M+1)^+$

1-{3-[(2,2-Dimethyl-5-morpholin-4-yl-1,4-dihydro-2H-pyrano-[4″,3″:4′,5′]pyrido[3′,2′:4,5] thieno[3,2-d]pyrimidin-8-yl)amino]-propyl}pyrrolidin-2-one (32). Obtained (80%) from compound

5 and 1-(3-aminopropyl)-pyrrolidin-2-one following the experimental procedure described for compound **13**. mp 215.9−216.7 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 1.3 (s, 6 H), 1.8 (m, 2 H), 1.9 (m, 2 H), 2.2 (t, J = 8.2 Hz, 2 H), 3.2 (m, 4 H), 3.3 (m, 2 H), 3.4 (m, 2 H), 3.5 (m, 4 H), 3.8 (m, 4 H), 4.7 (s, 2 H), 7.7 (t, J = 5.5 Hz, 1 H), 8.6 (s, 1 H). HPLC: 98.2%, t_R = 15 min, MS (ESI) 497 m/z $(M+1)^+$.

1-(3-{[5-Morpholin-4-yl)-2,2-dimethyl-1,2,3,4-tetrahydropyrimido[4′,5′:4,5]thieno[2,3-*c*]isoquinolin-8-yl]amino}propyl)pyrrolilydin-2-one (33). Obtained (81%) from compound **6** and 1-(3-aminopropyl)pyrrolidin-2-one following the experimental procedure described for compound **13**. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 1.12 (s, 6 H), 1.59−1.63 (m, 2 H), 1.81−1.93 (m, 2 H), 1.99−2.14 (m, 2 H), 2.48 (t, J = 8.22 Hz, 2 H), 2.77 (t, J = 6.46 Hz, 2 H), 3.25−3.32 (m, 4 H), 3.38−3.52 (m, 6 H), 3.66 (q, J = 6.13 Hz, 2 H), 3.85−3.92 (m, 4 H), 6.29 (t, J = 6.06 Hz, 1 H), 8.69 (s, 1 H). HPLC: 96.9%, t_R = 19 min, MS (ESI) 495 m/z $(M+1)^+$.

2,2-Dimethyl-5-morpholin-4-yl-*N*-(pyridin-3-ylmethyl)-1,4-dihydro-2*H*-pyrano[4″,3″:4′,5′]pyrido[3′,2′:4,5]thieno[3,2-*d*]pyrimidin-8-amine (34). Obtained (76%) from compound **5** and pyridin-3-ylmethylamine following the experimental procedure described for compound **13**. mp 260.6−261.7 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 1.32 (s, 6 H), 3.19 (m, 4 H), 3.50 (s, 2 H), 3.76 (m, 4 H), 4.70 (s, 2 H), 4.77 (d, J = 5.80 Hz, 2 H), 7.35 (dd, J = 7.63, 4.58 Hz, 1 H), 7.77 (m, 1 H), 8.39 (t, J = 5.80 Hz, 1 H), 8.46 (dd, J = 4.58, 1.53 Hz, 1 H), 8.59 (s, 1 H), 8.61 (d, J = 1.53 Hz, 1 H). HPLC: 98.3%, t_R = 14 min, MS (ESI) 463 m/z $(M+1)^+$.

2,2-Dimethyl-5-morpholin-4-yl-*N*-(pyridin-3-ylmethyl)-1,2,3,4-tetrahydropyrimido[4′,5′:4,5]thieno[2,3-*c*]isoquinolin-8-amine (35). Obtained (84%) from compound **6** and (pyridin-3-ylmethyl)amine following the experimental procedure described for compound **13**. mp 248.8−249.3 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 1.07 (s, 6 H), 1.55 (t, J = 5.80 Hz, 2 H), 2.76 (s, 2 H), 3.21 (s, 4 H), 3.36 (m, 2 H), 3.78 (s, 4 H), 4.77 (d, J = 5.80 Hz, 2 H), 7.36 (m, 1 H), 7.77 (d, J = 7.94 Hz, 1 H), 8.32 (d, J = 6.10 Hz, 1 H), 8.46 (d, J = 4.88 Hz, 1 H), 8.61 (m, 2 H). HPLC: 99.8%, t_R = 18 min, MS (ESI) 461 m/z $(M+1)^+$.

2,2-Dimethyl-5-morpholin-4-yl-*N*-(pyridin-4-ylmethyl)-1,4-dihydro-2*H*-pyrano[4″,3″:4′,5′]pyrido[3′,2′:4,5]thieno[3,2-*d*]pyrimidin-8-amine (36). Obtained (34%) from compound **5** and pyridin-4-ylmethylamine following the experimental procedure described for compound **13**. mp 238.0−239.7 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 1.32 (s, 6 H), 3.20 (m, 4 H), 3.50 (s, 2 H), 3.77 (m, 4 H), 4.71 (s, 2 H), 4.77 (d, J = 5.80 Hz, 2 H), 7.33 (d, J = 6.10 Hz, 2 H), 8.44 (t, J = 6.10 Hz, 1 H), 8.49 (m, 2 H), 8.56 (s, 1 H). HPLC: 97.8%, t_R = 12 min, MS (ESI) 463 m/z $(M+1)^+$.

2,2-Dimethyl-5-morpholin-4-yl-*N*-(pyridin-4-ylmethyl)-1,2,3,4-tetrahydropyrimido[4′,5′:4,5]thieno[2,3-*c*]isoquinolin-8-amine (37). Obtained (45%) from compound **6** and (pyridin-4-ylmethyl)amine following the experimental procedure described for compound **13**. mp 239.9−240.8 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 1.06 (s, 6 H), 1.54 (t, J = 5.49 Hz, 2 H), 2.76 (m, 2 H), 3.22 (s, 4 H), 3.38 (m, 2H), 3.78 (s, 4 H), 4.76 (d, J = 5.49 Hz, 2 H), 7.33 (d, J = 4.88 Hz, 2 H), 8.37 (t, J = 5.49 Hz, 1 H), 8.49 (d, J = 5.49 Hz, 2 H), 8.56 (s, 1 H). HPLC: 98.4%, t_R = 16 min, MS (ESI) 461 m/z $(M+1)^+$.

***N*-(2-Methoxybenzyl)-2,2-dimethyl-5-morpholin-4-yl-1,4-dihydro-2*H*-pyrano[4″,3″:4′,5′]thieno[3,2-*d*]pyrimidin-8-amine (38).** Obtained (74%) from compound **5** and 2-methoxybenzylamine following the experimental procedure described for compound **13**. ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 1.32 (s, 6 H), 3.20 (d, J = 4.58 Hz, 4 H), 3.76 (d, J = 4.58 Hz, 4 H), 3.85 (s, 4 H), 4.61−4.80 (m, 5 H), 6.87 (t, J = 7.48 Hz, 1 H), 7.01 (d, J = 7.63 Hz, 1 H), 7.15 (d, J = 7.32 Hz, 1 H), 7.23 (t, J = 7.78 Hz, 1 H), 8.20 (t, J = 5.65 Hz, 1 H), 8.54 (s, 1 H). HPLC: 99.3%, t_R = 21 min, MS (ESI) 490 m/z $(M+1)^+$.

***N*-(2-Methoxybenzyl)-2,2-dimethyl-5-morpholin-4-yl-1,2,3,4-tetrahydropyrimido[4′,5′:4,5]thieno[2,3-*c*]isoquinolin-8-amine (39).** Obtained (90%) from compound **6** and 2-methoxybenzylamine following the experimental procedure described for compound **13**. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 1.13 (s, 6 H), 1.50−1.68 (m, 4 H), 2.77 (t, J = 6.46 Hz, 2 H), 3.30 (d, J = 4.67 Hz, 4 H), 3.45 (s, 2 H), 3.89 (d, J = 4.94 Hz, 5 H), 4.88 (d, J = 5.77 Hz, 2 H), 5.16−5.39 (m, 1 H), 6.93 (d, J = 7.97 Hz, 2 H), 7.21−7.35 (m, 1 H), 7.39 (d, J = 7.69 Hz, 1 H), 8.75 (s, 1 H). HPLC: 98.3%, t_R = 11 min, MS (ESI) 427 m/z $(M+1)^+$.

2-[(2,2-Dimethyl-5-morpholin-4-yl-1,4-dihydro-2*H*-pyrano[4″,3″:4′,5′]pyrido[3′,2′:4,5]thieno[3,2-*d*]pyrimidin-8-yl)(2-morpholin-4-ylethyl)amino]ethanol (40). Obtained (41%) from compound **5** and 2-(2-morpholin-4-ylethylamino)-ethanol following the experimental procedure described for compound **13**. mp 111.9−112.6 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 1.3 (s, 6 H), 2.5 (m, 4 H), 2.6 (t, J = 6.8 Hz, 2 H), 3.2 (m, 4 H), 3.5 (s, 2 H), 3.6 (m, 4 H), 3.7 (m, 6 H), 3.9 (t, J = 6.1 Hz, 2 H), 3.9 (t, J = 6.8 Hz, 2 H), 4.7 (s, 2 H), 5.0 (t, J = 5.7 Hz, 1 H), 8.6 (s, 1 H). HPLC: 97.8%, t_R = 9 min, MS (ESI) 529 m/z $(M+1)^+$.

2-[(2,2-Dimethyl-5-morpholin-4-yl-1,2,3,4-tetrahydropyrimido-[4′,5′:4,5]thieno[2,3-*c*]isoquinolin-8-yl)(2-morpholin-4-ylethyl)-amino]ethanol (41). Obtained (57%) from compound **6** and 2-(2-morpholin-4-ylethylamino)ethanol following the experimental procedure described for compound **13**. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 1.1 (s, 6 H), 1.6 (t, J = 6.3 Hz, 2 H), 2.6 (s, 4 H), 2.8 (t, J = 6.3 Hz, 2 H), 2.9 (m, 2 H), 3.3 (m, 4 H), 3.5 (s, 3 H), 3.7 (m, 4 H), 3.9 (m, 4 H), 4.0 (m, 6 H), 8.6 (s, 1 H). HPLC: 96.8%, t_R = 12 min, MS (ESI) 527 m/z $(M+1)^+$.

2,2-Dimethyl-5-morpholin-4-yl-*N*-(2-morpholin-4-ylethyl)-N-(pyridin-3-ylmethyl)- 1,4-dihydro-2*H*-pyrano[4″,3″:4′,5′]pyrido-[3′,2′:4,5]thieno[3,2-*d*]pyrimidin-8-amine (42). Obtained (14%) from compound **5** and (2-morpholin-4-ylethyl)-pyridin-2-ylmethylamine following the experimental procedure described for compound **13**. mp 149.8−150.3 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 1.3 (s, 6 H), 2.4 (s, 6 H), 2.7 (t, J = 6.8 Hz, 2 H), 3.2 (m, 4 H), 3.5 (d, J = 8.7 Hz, 4 H), 3.8 (m, 4 H), 3.9 (t, J = 6.6 Hz, 2 H), 4.7 (s, 2 H), 5.2 (s, 2 H), 7.3 (dd, J = 7.5, 4.6 Hz, 1 H), 7.7 (d, J = 8.3 Hz, 1 H), 8.5 (m, 1 H), 8.6 (d, J = 1.7 Hz, 1 H), 8.6 (s, 1 H). HPLC: 96.6%, t_R = 10 min, MS (ESI) 576 m/z $(M+1)^+$.

Biology: General. PDE4 Enzyme Preparation. Yeast strains of *P. pastoris* overexpressing different recombinant PDE4 subtypes (PDE4B1, PDE4A4, and PDE4D3, Accession numbers L20966, L20965, and L20970, respectively) were inoculated in 500 mL flasks with 100 mL YPD (1 g yeast extract, 2 g peptone, 95 mL H_2O, and 5 mL 40% glucose) and grown at 30 °C under orbital shaking (150 rpm) for 48 h to ensure one reaching near OD600 = 2.0. Then, cells were harvested by centrifugation at 1500 rpm for 10 min at 20 °C. All supernatants were removed and pellets resuspended in the appropriate volume of SD (1.7 g yeast nitrogen base w/o amino acids and sulfate, 5 g $(NH_4)_2SO_4$, 30% galactose, 0.5% adenine sulfate, and 050 mg/mL L-lysine−HCl) to obtain an OD600 between 0.4 and 0.6 and then incubated with shaking at 30 °C until an OD600 of 2.0 was reached (usually 48 h).

Then, cells were centrifuged at 4000 rpm for 20 min at 4 °C and supernatants discarded. Pellets were resuspended in 100 mL of ice-cold sterile water and centrifuged at 4000 rpm for 20 min at 4 °C. Supernatants were discarded, and the wet weight of pellets was measured and 1.3 volume/weight of homogenization buffer, YHB (2 M KCl, 0.5 M EDTA, 1 M HEPES) supplemented with DTT and protease inhibitors added and then resuspended. Another centrifugation at 4000 rpm for 20 min at 4 °C was carried out. Supernatants were discarded and using a spatula yeast paste were transfered into a 10 mL syringe, and yeast "noodles" were made and placed into small plastic beakers filled with liquid nitrogen. The frozen yeast "noodles" can be stored at −80 °C until the day of extract preparation. Yeast "noodles" were poured into a cooled porcelain mortar full of liquid nitrogen and crushed under liquid nitrogen until yeast showed a powdery smooth consistency. Then, the yeast/liquid nitrogen suspension was poured into a plastic beaker and the remaining yeast powder scraped into a beaker with spatula. Dialysis buffer (1.4 volume/weight), YDB (2 M KCl, 0.5 M EDTA, 1 M HEPES, 100% glycerol, 1 M DTT, 0.25 M PMSF, 1 mg/mL leupeptin, and supplemented with DTT and

protease inhibitors) was then added to resuspended yeast powder and then centrifuged at 25 000 rpm for 2 h at 4 °C. The lipidic layer was removed; supernatants were recovered and transferred to dialysis tubing and dialyzed overnight against 500 mL YDB.

After dialysis, supernatants were transferred to microtubes in small aliquots (100 μL) and quick-frozen and stored at −80 °C; also, protein concentration was determined by the method of Bradford with the Bio-Rad protein assay kit and using bovine serum albumin (BSA) as a standard.

PDE4 Activity Determination. PDE4 activity from various human recombinant PDE4 subtypes (PDE4B1, PDE4A4, and PDE4D3) was monitored by measuring the hydrolysis of [^{3}H]-cAMP to [^{3}H]-AMP using a PDE-SPA kit from Amersham International as previously described.[17] Enzyme extracts (~4 μg of protein) were incubated in "low binding" plates (Costar 3604) for 60 min at room temperature. The assay mixture (80 μL) contains 15 nM [^{3}H]-cAMP (1 μCi/mL) in the assay buffer (50 mM Tris pH 7.5, 8.3 mM $MgCl_2$, 1.7 mM EGTA) and 10 μL of test compound. These compounds were resuspended in DMSO (the final DMSO concentration 5% (v/v)) at a stock concentration of 1 mM. The compounds were tested at different concentrations varying from 10 μM to 10 pM to calculate an IC_{50}. These dilutions were done in 96-well plates. In some cases, plates containing diluted compounds were frozen before being assayed. In these cases, the plates were thawed at room temperature and agitated for 15 min.

Hydrolysis of [^{3}H]-cAMP was initiated by adding 10 μL of a solution containing PDE4 enzyme, and the plate was then incubated under agitation at room temperature. The reaction was stopped after 60 min (with ~10−20% substrate conversion) by addition of 50 μL Phosphodiesterase Scintillation Proximity Assay (SPA) Beads. All reactions were carried out in duplicate. [^{3}H]-AMP, captured by the SPA beads, was determined by counting the plates in a Wallac-Microbeta Trilux scintillation counter 1 h after addition of the beads, although the signal was quite stable, and samples may be counted from 1 to 48 h after bead addition.

LPS Induced TNF-α in Human Whole Blood (HWB-TNF-α). Human whole blood of healthy donors was collected in 50 mL Falcon tubes with heparin (5000 units/mL, Heparin Mayne 5%, MAYNE PHARMA). LPS (lipopolysaccharide from *Escherichia coli*, Sigma, St. Louis, MO) dissolved in PBS (Dulbecco's phosphate buffered saline, without calcium and magnesium chloride Sigma, St. Louis, MO) was added to the tubes to give a final concentration in the assay of 1 μg/mL and preincubated at 37 °C for 10 min with rocking. Increasing concentrations of different inhibitors (2 μL), dissolved in 100% DMSO, were added to the 96-well plates and 200 μL of blood containing LPS (except for controls) then distributed into wells, plates were shaken for 1−2 min, sealed with aluminum foil lid (Beckman Coulter), and then incubated for 24 h at 37 °C under agitation in KelvitronT (Heraeus Instruments).

After the 24 h period, plates were placed on ice, 50 μL of PBS added, and the reaction was stopped by centrifugation of plates at 2000 rpm (800*g*) at 4 °C for 15 min. Serum obtained was then subjected to ELISA or kept at −80 °C until use.

TNF-α Determination. The quantification of TNF-α in human serum was performed using commercial ELISA kit (DuoSet) obtained from R&D Systems, Inc., and following the manufacturer's instructions.

Plate Preparation: First, R&D DuoSet ELISA 96-well microplates were coated with 4.0 μg/mL mouse antihuman TNF-α diluted in PBS, overnight at room temperature. After washing, plates were then blocked with PBS containing 1% BSA for a minimum of 1 h at room temperature and then washed.

Assay Procedure: 100 μL of samples or standard was added and incubated at 2 h at room temperature. After washing (ELX406 Select, BIO-TEK), biotinylated anti-hTNF-α antibody was added and incubated at room temperature for 2 h, followed by incubation with streptavidin−peroxidase for 20 min. Detection of bound hTNF-α was carried out with 100 μL of substrate solution (H_2O_2 and tetramethylbenzidine) followed by measurement at 450 nm in a SPECTRA max Plus (Molecular Devices).

These experiments were performed 2−3 times using the same experimental design. Duplicates from each series of experiments were averaged and expressed as hTNF-α levels in pg/mL.

In Vitro Rat, Mouse, and Human Plasma Protein Binding. Plasma protein binding is typically determined using the ultrafiltration technique. The NCE solution is first prepared by adding an aliquot of concentrated NCE (typically 5 μL of 100 μg/mL in DMSO) to 1 mL of plasma to achieve the desired final concentration of 2.5 μg/mL and 1% organic solvent. The plasma is mixed and incubated for 2 h at 37 °C in a shaker water bath (85 U/min).

Each solution is then loaded onto the Centrifree filter (YM-30, Millipore) and centrifuged at 2000 *g* for 15 min at 37 °C. An aliquot of the ultrafiltrate is diluted (1:1) with acetonitrile/water and analyzed by HPLC-MS. A blank incubation without plasma is performed in parallel to account for the unspecific binding. Samples are quantified using 25 and 250 ng/mL standard points.

Study of Transport through Caco-2 Cell Barrier (Active or Passive Transport). To study the transport characteristics of a new NCE and account for passive or active transport, the Caco-2 cells are used to study permeability from the apical to basolateral side (AB transport), as well as basolateral to apical side (BA transport). Passage numbers from 20 to 40 are used for transport studies. Experiments are performed using monolayers grown with the Biocoat HTS-Caco-2 Assay System (Becton & Dickinson) according to the protocol of the kit. 24-well plate format from BIOCAT with PET membranes (1 μm pore size) with a layer of fibrillar collagen are also used for the experiments. Briefly, Caco-2 cells are seeded at 500 000 cell/well in Mito+ Serum Extender supplemented medium incubated for 56 h. Then, medium is changed to Mito+ supplemented Entero-STIM Differentiation Media (ESM+) and incubated for another 48 h. Then, ESM+ is renewed by fresh ESM+ and incubated for 24 h extra. Six days postseeding cells are used to perform transport experiments. The NCE (solution in HBSS supplemented buffer and maximum of 1% DMSO) or supplemented HBSS buffer (10 mM Hepes and 25 mM glucose at pH 7.4) will be placed in the apical or basolateral side as indicated in the table below and according to the AB or BA transport study. After a 3 h incubation period, samples are collected, diluted with methanol (1:1), and analyzed by HPLC-UV. [^{3}H]Mannitol at 115 nM is used as a positive control of the membrane integrity. Outcome of the assay obtained include apparent permeability for directions (PappAB and PappBA), percentage of absorption, material balance after 3 h, and stability at 37 °C after 3 h.

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