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Modulation of cAMP-Specific PDE without Emetogenic Activity: New Sulfide-Like PDE7 Inhibitors

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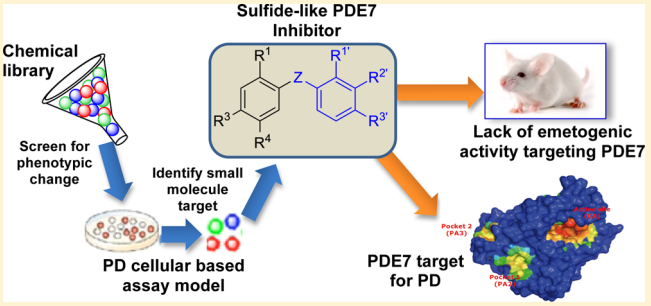
Supporting Information

ABSTRACT: A forward chemical genetic approach was followed to discover new targets and lead compounds for Parkinson's disease (PD) treatment. By analysis of the cell protection produced by some small molecules, a diphenyl sulfide compound was revealed to be a new phosphodiesterase 7 (PDE7) inhibitor and identified as a new hit. This result allows us to confirm the utility of PDE7 inhibitors as a potential pharmacological treatment of PD. On the basis of these data, a diverse family of diphenyl sulfides has been developed and pharmacologically evaluated in the present work. Moreover, to gain insight into the safety of PDE7 inhibitors for human chronic treatment, we evaluated the new compounds in a surrogate emesis model, showing nonemetic effects.



INTRODUCTION

Parkinson's disease (PD) is a progressive neurodegenerative disorder that is characterized by the inability to initiate, execute, and control movements. Common symptoms of PD patients are tremor, rigidity, bradykinesia, akinesia, postural reflex abnormalities, gait disturbance, etc.[1] In addition to motor difficulties, parkinsonian patients experience bouts of mild depression and irritability as well as cognitive disturbances defined by memory and attention perturbations.[2] PD is a disease of high incidence, especially in high-income countries, where progressive aging of the population is one of the main risk factors. With the partial exception of juvenile parkinsonism, development of PD emerges during the fifth and sixth decade, affecting 1 in 2000 people, and the prevalence is consistently higher in men than in women. This incidence increases to 1 in 500 people older than 65 years.[3] Today, there are more than one million PD patients in the USA and double this figure worldwide, thus representing high social and healthcare costs.[4]

As a neurodegenerative disease, PD shares with these devastating pathologies an unknown etiology, the progressive destruction of specific areas of the brain, and the lack of an effective treatment. Currently, the etiology of PD is not well understood but is likely to involve both genetic and environmental factors.[5] Neuropathologically, PD is characterized by the loss of dopamine-producing neurons in the substantia nigra pars compacta of the midbrain, followed by striatal dopamine depletion and indirectly by cortical dysfunction.[6]

Among the therapeutic agents for the palliative treatment of PD, L-3,4-dihydroxyphenylalanine (L-dopa) is the drug most frequently used. It ameliorates parkinsonian-associated motor impediments but is relatively inefficient in alleviating the affective and cognitive symptoms of the disease.[7] However, long-term administration of L-dopa causes important side effects, such as dyskinesia, that contribute to lower quality of life of patients.[8] Therefore, there is an urgent need for the development of new therapeutic agents for PD or new concomitant agents to avoid the high-dose administration of L-dopa.[9]

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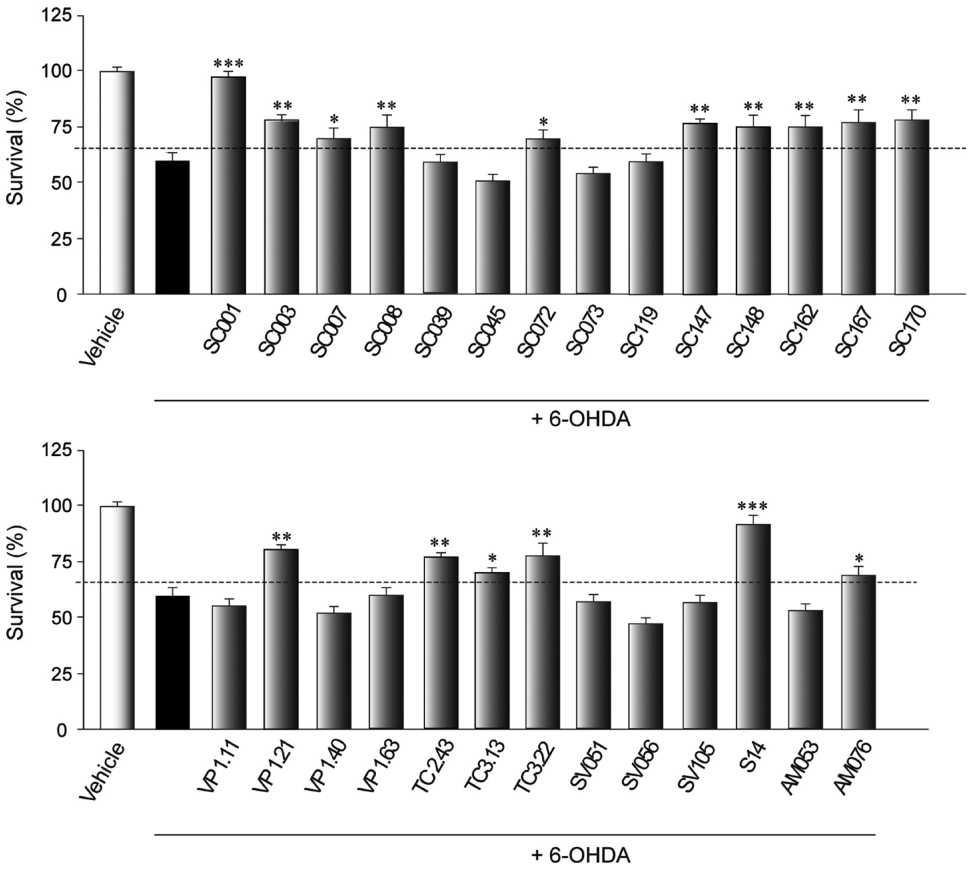

Figure 1. Phenotypic screening of our in-house small molecule chemical library. Typical plot obtained in our chemical genetic approach to discover new pharmacological targets for PD. Results for 27 different compounds among a data set of 450 small molecules tested, including compound **2** (named as **SC072**), are depicted. SH-SY5Y cells were exposed to 6-OHDA [35 μM] during 24 h in the presence or absence of the small heterocyclic compounds [10 μM]. The number of viable cells was measured by MTT assay. Each data point represents the mean ± SD of four replications in three different experiments. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ versus 6-OHDA treated cultures (from ref 14).

In this context, we have reported the development of PDE7 inhibitors as a new approach to be explored for the treatment of neurological disorders due to increasing levels of cAMP[10] and specifically PDE7 inhibition by hindering dopaminergic cell death and glial activation in a toxicity-based animal model of PD. A heterocyclic small-molecule inhibitor of PDE7 that was discovered in our research group, the 4-oxo-3-phenyl-2-thioxo-1,2,3,4-tetrahydroquinazoline (**1**),[11] conferred significant neuronal protection against different insults both in the human dopaminergic cell line SH-SY5Y and in primary rat mesencephalic cell cultures. Quinazoline **1** treatment also reduced microglial activation, protected dopaminergic neurons, and improved motor function in the lipopolysaccharide rat model of Parkinson's disease. Moreover, quinazoline **1** neuroprotective effects were abolished by blocking the cAMP signaling pathways that operate through cAMP-dependent PKA. These results showed for the first time that inhibition of the PDE7 enzyme leads to dopaminergic neuronal protection and, therefore, its inhibitors may exert useful therapeutic actions in patients with PD.[12,13] Furthermore, the PDE7 inhibitor **1** (also known as **S14**) is currently in regulatory toxicological studies to enter clinical trials for PD.

With the aim of discovering new targets for PD, a chemical genetic approach involving the use of small molecules as pharmacological tools was implemented in our group. First, a screening of our in-house chemical library on a well-established cellular model of PD was done, followed by a detailed pharmacological analysis of the hits, which allowed us to determine new targets for PD treatment (Figure 1). Thus, by analyzing the cell protection produced by the 5-(3-chlorophenyl)-1,3-diphenyl-1,2,4-benzotriazepine (**SC001**), we could demonstrate that GSK-3 is a good target for PD treatment.[14] Here, we describe data obtained for the *N*-[2-(5-chloro-2-nitrophenylthio)phenyl]acetamide (**2**), which was revealed to be a new PDE7 inhibitor. The study here reported allows us to confirm the utility of PDE7 inhibitors as a potential pharmacological treatment of PD.[12] Moreover, to gain insight into the safety of PDE7 inhibitors, we here evaluate the new compounds in a well-established surrogate emesis model,[15] showing that modulation of PDE7 avoids gastrointestinal side effects such as those produced by PDE4 inhibitors. Human emesis, which was found in clinical development of several PDE4 inhibitors, has jeopardized their development and has ruled out some promising candidates from reaching the pharmaceutical market.

RESULTS AND DISCUSSION

Discovery of Sulfide-Like PDE7A Inhibitors. Our initial screening was based on chemical diversity. From the chemical structures collected in our in-house chemical library, we selected 450 diverse small heterocyclic compounds for the first cell-based phenotypic screening. We analyzed all the compounds that resulted in more than 65% cell survival in our assay conditions (6-hydroxydopamine (6-OHDA)-induced

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Table 1. PDEs Panel from Sulfide 2[a]

	PDE7A	PDE3A	PDE4D	PDE4B
2	IC_{50} = 2.1 ± 0.2 μM	10 ± 3% @ 10 μM	10 ± 2% @ 10 μM	11 ± 7% @ 10 μM

[a]Values represent the mean ± standard deviation of two independent experiments (n = 2) with duplicate measurements.

human neuroblastoma cell death, see Experimental Section). Here, we report results derived from the analyses of the hit compound **2**. In the forward chemical genetics approach, one of the key steps is the identification of the target that produced the phenotypic change. For that reason, we evaluated the diphenyl sulfide **2** in our internal biological assays to determine its potential target. Thus, we run ORAC experiments to look for antioxidant activity, or potential inhibition of some protein kinases, including GSK-3β, CK1δ, CK1ε, LRRK2, and ROCK, and PDEs inhibition, such as PDE7A, PDE3A, PDE4B, and PDE4D. In all cases, we used a fixed concentration of compound **2** of 10 μM, and we evaluated the percentage of inhibition. When more than 50% inhibition was obtained, the dose–response curve was determined and the IC_{50} value was calculated. For sulfide **2**, no antioxidant activity or protein kinase inhibition was found in these experimental conditions. However, a consistent and rather selective PDE7A inhibition was found in human isoforms (Table 1), and thus, we can asses the neuroprotection in our PD cell-based model due to PDE7 inhibition, confirming our previous data with quinazoline-like inhibitors.[12]

In a second step, a small, focused subset of molecules from our chemical library was selected to confirm this specific biological activity. Thus, seven more diphenyl sulfide-like compounds similar to the hit were evaluated against PDE7A (Figure 2), showing that almost all the evaluated compounds inhibited PDE7A in the same range as the hit although with lower potency.

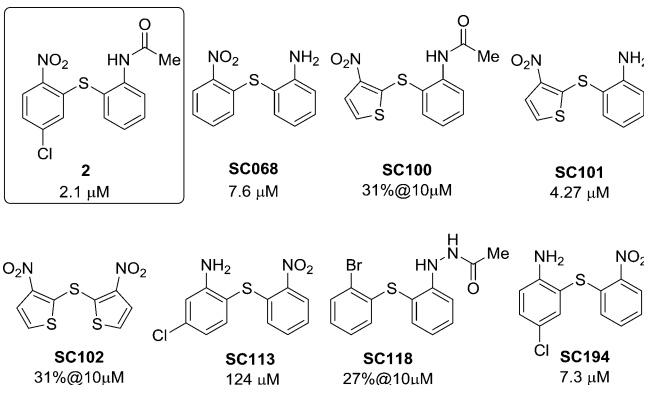

Figure 2. Small focused subset of sulfide **2**-related compounds selected from our chemical library and evaluation as PDE7 inhibitors.

Optimisation of PDE7 Enzyme Potency. Given its inhibition against PDE7A, its promising behavior in vitro, and its chemical diversity from previously described inhibitors of PDE7, compound **2** was selected for further biological activity optimization. **2** has two phenyl rings connected by a sulfide linker. We carried out a medicinal chemistry program to increase the potency of the hit, consisting of the structural modification of the substituents attached to both phenyl rings and changes in the nature of the linker and the substituents of the phenyl rings. Thus, other linkers, an ether moiety and different heterocycles, such as pyridine or thiophene, have been assayed (Schemes 1–6).

In general, almost all the new synthesized compounds were obtained by nucleophilic substitution of aryl halides and the corresponding thiol or alkoxy derivatives. These reactions were carried out using the base and solvent that was most convenient in each case (Schemes 1, 2, and 5–6). As indicated in Scheme 1, optimization of the synthetic procedure of compounds **14** and **28** was carried out and microwave irradiation was used to increase yields and reduce reaction times. In fact, sulfides **3**–**14** were synthesized in dichloromethane and in the presence of potassium carbonate as previously described.[16] The use of microwave irradiation brought a significant decrease of the reaction time with respect to the reported procedure by conventional heating (21 h). However, due to the frequent dimerization of the thiol reagent, we tried other reaction conditions such as ethanol/sodium acetate.[17] In this case, we could observe a significant reduction in the dimerization product, although no significant improvement in yields (optimization of synthesis of compound **14**). Moreover, for further optimization, we decided to try other solvents with higher boiling points such as acetonitrile or *N*,*N*-dimethylformamide. As a result of the increasing temperature, we were able to significantly reduce the reaction time as well as to increase the yields (optimization of synthesis of compound **28**).

To introduce a new point of diversity, compounds **28** and **8** were *N*-acetylated (Scheme 3) and the phenyl nitro groups were converted into amino groups by treatment with a reducing agent such as tin(II) chloride (Scheme 4).

In Vitro Evaluation of PDE7 Inhibition and Binding Mode Studies. The new derivatives (**3**–**58**) were tested for their inhibitory potencies against PDE7A1 using the recombinant human isoenzyme as described in the Experimental Section. All the compounds were tested at a fixed concentration of 10 μM, and the experiments were performed in duplicate. The percentage of inhibition of PDE7A1 for all the compounds at this concentration is shown in Table 2. When the percentage of PDE7A1 inhibition was greater than 50%, the dose–response curve was determined and the IC_{50} values were calculated (Table 2). Some of these new compounds presented IC_{50} values in the low micromolar range and thus are good hits to be further explored as pharmacological agents.

For better characterization of the enzymatic inhibition of this new family of PDE7 inhibitors, a kinetic study in which cAMP concentration was varied was performed with two of the most active compounds (**28** and **42**). Double-reciprocal plotting of the data is depicted in Figure 3. The intercept of the plot in the horizontal axis (1/[cAMP]) changes when the compound concentrations increase, whereas the intercept in the vertical axis ($1/V$) does not change. These results suggest that diphenyl-sulfide derivatives act as competitive inhibitors of cAMP in their binding to PDE7, targeting the catalytic binding site.

To gain insight into the binding mode of the sulfide-like inhibitors, docking experiments were carried out with the docking program Glide (Schrodinger Inc.) according to the procedure described in the Experimental Section for com-

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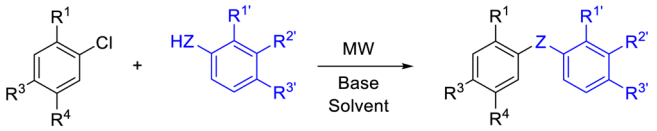
Scheme 1. General Procedure for the Preparation of Compounds 3−36



Comp.	R^1	R^3	R^4	Z	$R^{1'}$	$R^{2'}$	$R^{3'}$	Base	Solvent	Temp (°C)	Time (min)	Yield (%)
3	NO_2	Cl	Cl	S	NH_2	H	H	K_2CO_3	CH_2Cl_2	80	90	52
4	CF_3	NO_2	H	S	NH_2	H	H	K_2CO_3	CH_2Cl_2	80	90	65
5	Cl	NO_2	H	S	NH_2	H	H	K_2CO_3	CH_2Cl_2	80	90	40
6	NO_2	F	H	S	NH_2	H	H	K_2CO_3	CH_2Cl_2	80	90	51
7	CF_3	NO_2	H	S	H	H	NH_2	K_2CO_3	CH_2Cl_2	100	45	53
8	Cl	NO_2	H	S	H	H	NH_2	K_2CO_3	CH_2Cl_2	100	45	45
9	NO_2	H	Cl	S	H	H	NH_2	K_2CO_3	CH_2Cl_2	100	45	77
10	H	NO_2	Cl	S	H	H	NH_2	K_2CO_3	CH_2Cl_2	100	45	61
11	NO_2	H	Cl	S	H	H	Br	K_2CO_3	CH_2Cl_2	80	90	32
12	NO_2	H	Cl	S	Br	H	H	K_2CO_3	CH_2Cl_2	80	90	16
13	Cl	NO_2	H	S	Br	H	H	K_2CO_3	CH_2Cl_2	80	90	40
14	NO_2	H	Cl	S	NH_2	H	H	K_2CO_3	CH_2Cl_2	80	90	32
								AcONa	EtOH	85	120	50
15	NO_2	Cl	H	S	NH_2	H	H	AcONa	EtOH	85	120	60
16	NO_2	Cl	H	S	H	NH_2	H	AcONa	EtOH	85	120	32
17	Cl	NO_2	H	S	H	NH_2	H	AcONa	EtOH	85	120	77
18	NO_2	F	H	S	H	H	NH_2	AcONa	EtOH	85	120	54
19	NO_2	Cl	Cl	S	Br	H	H	AcONa	EtOH	85	90	17
20	NO_2	Cl	H	S	Br	H	H	AcONa	EtOH	85	90	70
21	Cl	NO_2	H	S	H	H	Br	AcONa	EtOH	85	90	57
22	NO_2	Cl	H	S	H	H	Br	AcONa	EtOH	85	90	85
23	Cl	NO_2	H	CH_2S	Br	H	H	AcONa	EtOH	85	90	32
24	NO_2	Cl	H	CH_2S	Br	H	H	AcONa	EtOH	85	90	45
25	Cl	NO_2	H	S	^{i}Pr	H	H	AcONa	EtOH	85	90	48
26	Cl	NO_2	H	S	H	H	^{i}Pr	AcONa	EtOH	85	90	30
27	NO_2	Cl	H	S	H	H	^{i}Pr	AcONa	EtOH	85	90	60
28	NO_2	Cl	H	S	H	H	NH_2	AcONa	EtOH	85	90	35
								Cs_2CO_3	AcCN	130	8	58
29	NO_2	Cl	H	S	H	H	NO_2	Cs_2CO_3	AcCN	130	8	49
30	Cl	NO_2	H	S	H	H	NO_2	Cs_2CO_3	AcCN	130	8	78
31	NO_2	Cl	H	S	H	H	H	Cs_2CO_3	AcCN	130	8	66
32	Cl	NO_2	H	S	H	H	H	Cs_2CO_3	AcCN	130	8	45
33	NO_2	Cl	H	O	H	H	NH_2	K_2CO_3	DMF	150	10	40
34	Cl	NO_2	H	O	H	H	NH_2	K_2CO_3	DMF	150	10	54
35	Cl	NO_2	H	O	H	H	4-NHAc	K_2CO_3	DMF	150	10	78
36	NO_2	Cl	H	O	H	H	4-NHAc	K_2CO_3	DMF	150	10	72

pounds **28** and **42**. Considering the kinetic studies results, the docking studies were focused on the cAMP binding site. Regarding the sulfide **28**, the binding mode shows a weak aromatic $\pi-\pi$ interaction of the poor-electron ring with Phe416 (Figure 4A). Moreover, the amine group present in the second phenyl moiety may play a key role for the activity because it is able to establish hydrogen bond interactions with oxygens from Thr321 and Asp362, which could also explain the loss of

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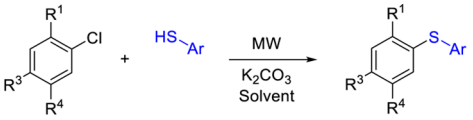
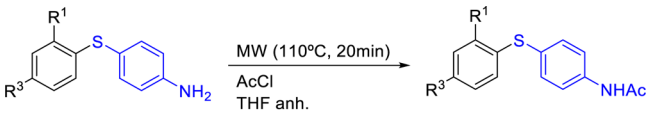
Scheme 2. General Procedure for the Preparation of Compounds 37−45



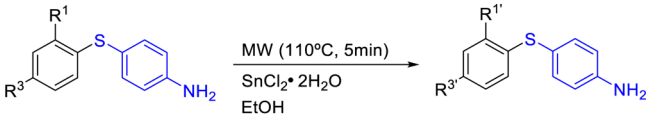
Comp.	R^1	R^3	R^4	Ar	Solvent	Temp. (ºC)	Time (min)	Yield (%)
37	Cl	NO_2	H	2-thienyl	THF	40	30	80
38	NO_2	Cl	H	2-thienyl	THF	40	30	92
39	NO_2	H	Cl	2-py	DMF	150	10	15
40	NO_2	Cl	H	2-py	DMF	150	10	92
41	Cl	NO_2	H	2-py	DMF	150	10	76
42	Cl	NO_2	H	4-py	DMF	150	10	52
43	NO_2	H	Cl	4-py	DMF	150	10	45
44	NO_2	Cl	H	4-py	DMF	150	10	88
45	NO_2	$COCH_3$	H	4-py	DMF	150	10	40

Scheme 3. General Procedure for the Preparation of Compounds 46 and 47



Comp.	R^1	R^3	Yield (%)
46	NO_2	Cl	70
47	Cl	NO_2	70

Scheme 4. General Procedure for the Preparation of Compounds 48 and 49

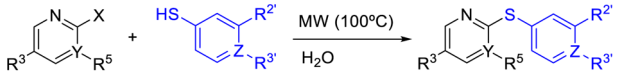


Comp.	R^1	R^3	$R^{1'}$	$R^{3'}$	Yield (%)
48	NO_2	Cl	NH_2	Cl	78
49	Cl	NO_2	Cl	NH_2	76

activity of compounds **22** and **27**, which have a bromine atom and an isopropyl group, respectively, in this position.

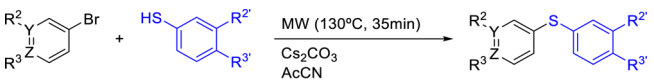
Regarding derivatives with 4-pyridine rings, such as compound **42** (Figure 4B), docking studies revealed the importance of the 4-pyridine ring for the activity, not only because of the aromatic interaction with Phe384 but also because the hydrogen bond formation with Asn260, which could also explain the higher affinity of 4-pyridine compounds

Scheme 5. General Procedure for the Preparation of Compounds 50−54



Comp.	R^3	R^5	Y	X	$R^{2'}$	$R^{3'}$	Z	Time (min)	Yield (%)
50	Me	NO_2	C	Cl	H	-	N	30	76
51	H	-	N	Cl	H	-	N	30	30
52	Me	NO_2	C	Cl	H	Ph	C	30	55
53	H	H	C	Br	H	Ph	C	30	70
54	Me	NO_2	C	Cl	NH_2	H	C	30	50

Scheme 6. General Procedure for the Preparation of Compounds 55−58



Comp.	R^2	R^3	Y	Z	$R^{2'}$	$R^{3'}$	Yield (%)
55	H	-	C	N	H	H	63
56	H	-	C	N	H	OMe	53
57	H	-	C	N	NH_2	H	33
58	-	H	N	C	H	OMe	58

in comparison with the 2-pyridine ones. Both PDE7 inhibitors, **28** and **42**, are able to interact with the conserved Gln413.

When comparing the binding modes of **28** and **42**, it is important to mention that the $\pi-\pi$ interaction between Phe416 and one aromatic ring of the sulfide derivative is conserved in both cases. However, the location of the second aromatic ring is completely different, probably due to the butterfly shaped sulfur bridge, which allows the movement of the ring looking for suitable interactions while maintaining the $\pi-\pi$ stacking with Phe416.

Moreover, PDE3 and PDE4 inhibition of compounds with an IC_{50} (PDE7A) ≤ 1.5 μM was calculated in order to assess safety of these potential drug candidates in further development. PDE3 and PDE4 inhibition in humans has been previously related with cardiotoxic[18] and emetogenic[19] side effects, respectively (Table 3), and they should be avoided in future chronic human treatment. The results show that diphenylsulfide derivatives are inhibitors of the two PDE7 isoforms, PDE7A and PDE7B, both of which are expressed in the brain, while in general these compounds are rather selective regarding PDE3 and PDE4. They do not present PDE3A inhibition at the concentration assayed, while the inhibition of some PDE4 isoforms is at least 1 order of magnitude greater than that of PDE7, if any. The most active PDE7A sulfide-type inhibitors, compounds **28** and **42**, were also evaluated for inhibition of PDE1A, PDE5, PDE8A, PDE9A, and PDE10A at 10 μM. Compound **28** inhibited these enzymes with a percentage of inhibition of 36.0 ± 1.0% (PDE1A), 28.0 ± 3.0% (PDE5), 1.2

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Table 2. PDE7A1 Inhibition of Compounds (3−58) Together with References Compounds BRL50481, Quinazoline 1, and Sulfide 2[a]

compd	PDE7A % inh @ 10 μM	PDE7A IC_{50}(μM)	compd	PDE7A % inh @ 10 μM	PDE7A IC_{50}(μM)
BRL50481	99 ± 2	0.09 ± 0.02	**30**	1 ± 2	
1	76 ± 2	4.68 ± 0.23	**31**	87 ± 1	1.50 ± 0.02
2	58 ± 2	2.10 ± 0.32	**32**	74 ± 1	2.20 ± 0.13
3	74 ± 4	6.48 ± 1.06	**33**	70 ± 1	3.30 ± 0.71
4	42 ± 6		**34**	77 ± 1	2.20 ± 0.10
5	60 ± 3	8.77 ± 1.34	**35**	4 ± 2	
6	38 ± 4		**36**	17 ± 1	
7	63 ± 4	9.27 ± 0.68	**37**	69 ± 2	5.20 ± 1.10
8	83 ± 1	3.97 ± 0.35	**38**	81 ± 1	1.60 ± 0.36
9	71 ± 1	2.20 ± 0.10	**39**	56 ± 2	7.98 ± 1.23
10	79 ± 2	1.47 ± 0.22	**40**	69 ± 2	3.60 ± 0.11
11	36 ± 1		**41**	69 ± 1	1.29 ± 0.64
12	69 ± 4	1.01 ± 0.10	**42**	85 ± 1	0.18 ± 0.09
13	59 ± 1	2.14 ± 0.63	**43**	80 ± 2	1.88 ± 0.21
14	66 ± 13	5.45 ± 1.17	**44**	91 ± 2	0.72 ± 0.06
15	69 ± 1	5.75 ± 1.36	**45**	56 ± 1	3.80 ± 0.63
16	68 ± 1	5.69 ± 0.41	**46**	7 ± 3	
17	78 ± 1	3.20 ± 0.83	**47**	26 ± 1	
18	64 ± 1	6.18 ± 0.21	**48**	13 ± 1	
19	49 ± 2	2.88 ± 0.17	**49**	46 ± 1	6.50 ± 1.03
20	79 ± 1	0.99 ± 0.05	**50**	27 ± 4	
21	23 ± 6		**51**	12 ± 1	
22	11 ± 1		**52**	2 ± 1	
23	33 ± 2		**53**	32 ± 3	
24	31 ± 2		**54**	3 ± 2	
25	17 ± 4		**55**	44 ± 1	
26	15 ± 2		**56**	18 ± 3	
27	10 ± 3		**57**	36 ± 4	
28	81 ± 1	0.37 ± 0.07	**58**	17 ± 1	-
29	6 ± 2				

[a]Values represent the mean ± standard deviation of two independent experiments (n = 2) with duplicate measurements.

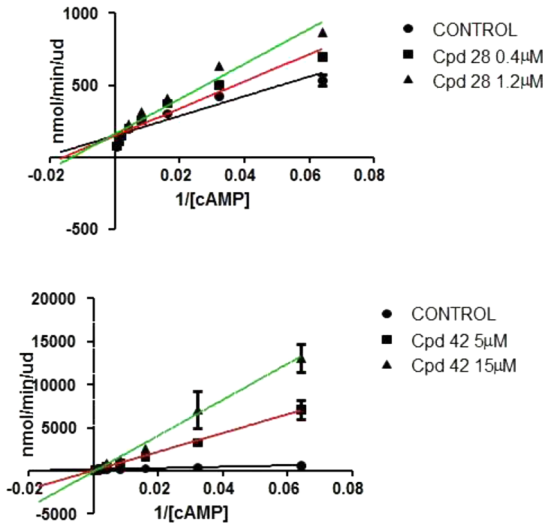

Figure 3. Studies of the PDE7A1 inhibition in the absence (control) and in the presence of two different concentrations of the sulfide derivatives **28** and **42** incubating different cAMP concentrations and measuring AMP formation. Data represent the mean ± SEM (vertical bars) of triplicate measurements.

± 0.8% (PDE8A), 7.0 ± 1.0% (PDE9A), and 57.5 ± 3.4% (PDE10A), and compound **42** with 39.0 ± 4.0% (PDE1A), 21.0 ± 1.0% (PDE5), 2.4 ± 1.1% (PDE8A), 1.0 ± 1.0% (PDE9A), and 29.4 ± 5.6% (PDE10A). These results show that these compounds can be considered fairly good hits as selective PDE7 inhibitors. Finally, it is worth mentioning that cAMP response element-binding protein (CREB) is a well-known target of the cAMP signaling pathway, and the enhancement of cAMP levels leads to an increase in its phosphorylation state. Thus, as a surrogate marker of the cAMP increase induced by our sulfide-like PDE7 inhibitors, we analyzed the phosphorylation levels of CREB in SH-SY5Y cultures after treatment with the compounds **28** and **42**. The use of an antiserum that does not discriminate between CREB and p-CREB indicated that the total levels of CREB were not affected by the treatments. Figure 5 clearly shows that the treatment with these compounds resulted in an increase in the phosphorylation state of CREB as a direct consequence of the up-regulation of cAMP levels.

Effects of New Sulfide-Like PDE7A Inhibitors on Cell Viability in SH-SY5Y Cells. Our next step was to explore the biological profile of the new derivatives. First, we tested if the new PDE7A inhibitors are able to protect the SH-SY5Y cell line from cell death induced by the toxin 6-OHDA, a well-established cell model for Parkinson's disease. The ability of the selected compounds (**10**, **12**, **20**, **28**, **31**, **41**, **42**, and **44**, all with an IC_{50} ≤1.5 μM) to protect the human neuroblastoma cell line from cell death was analyzed (Figure 6). As expected, treatment of SH-SY5Y cells with 6-OHDA resulted in significant cell death, while compounds **20**, **28**, and **44** significantly rescued

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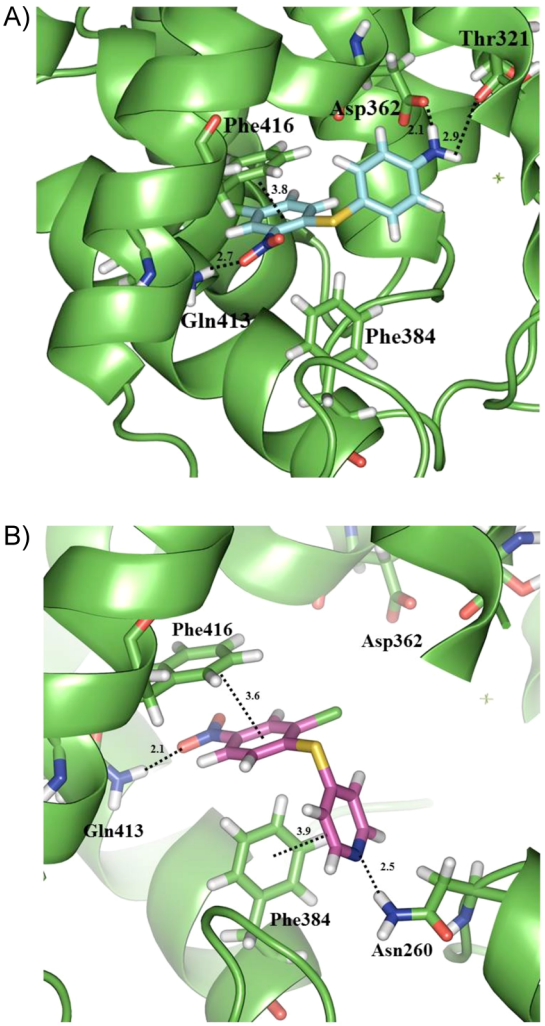

Figure 4. Proposed binding mode for compound **28** (A) and **42** (B) (PDB code 1ZKL) showing relevant interactions with nearby residues. Distances are given in Å.

human dopaminergic cells from death. Of note, derivative **28** increased cell survival with the same efficiency as two references compounds: the standard PDE7 inhibitor BRL50481 and the quinazoline-type PDE7 inhibitor **1**, currently in pharmaceutical development for PD.[12,20]

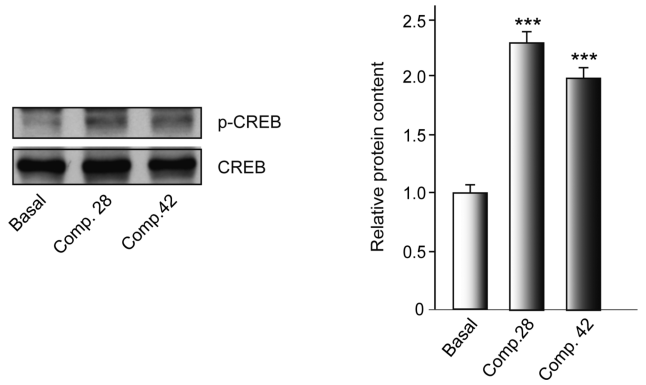

Figure 5. Effects of compounds **28** and **42** on phosphorylation state of the cAMP response element-binding protein (CREB). Representative Western blot showing the levels of p-CREB on SH-SY5Y after treatment with compounds **28** and **42** (10 μM). Results are mean values ± SD from three independent experiments. Quantification analysis is shown. ***$P \leq 0.001$ versus nontreated (basal) cultures.

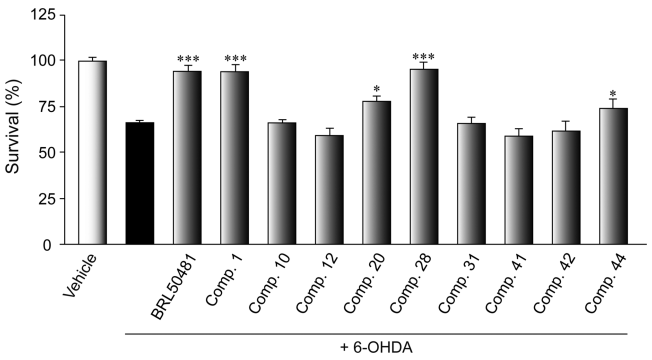

Figure 6. SH-SY5Y cells were exposed to 6-OHDA [35 μM] during 16 h in the presence or absence of the PDE7A inhibitors at 10 μM. The number of viable cell was measured by MTT assay. Values represent the means ± SD from three different experiments. ***$p \leq 0.001$, *$p \leq 0.05$ versus 6-OHDA-treated cells.

Anti-inflammatory Effects of New Sulfide-Like PDE7A Inhibitors. To further evaluate the role of PDE7A inhibitors in inflammatory reactions of neuronal cells, we performed experiments in different cell-based assays. We used primary cultures of astrocytes and microglia treated with lipopolysaccharide (LPS), a potent inflammatory agent. The potential anti-inflammatory activity of the selected PDE7A inhibitors was tested by evaluating the production of nitrites from primary

Table 3. PDEs Profiling from Those Phenylsulfides with an IC_{50} (PDE7A) ≤ 1.5μM (BRL50481[a] and Rolipram[b] Are Included As Standard References)[c]

compd	PDE7A IC_{50} (μM)	PDE7B %inh @10 μM	PDE7B IC_{50} (μM)	PDE3A %inh @10 μM	PDE4D %inh @10 μM	PDE4D IC_{50} (μM)	PDE4B %inh @10 μM	PDE4B IC_{50} (μM)
10	1.47 ± 0.17	42 ± 2		22 ± 2	3 ± 1		2 ± 1	
12	1.01 ± 0.23	71 ± 1	2.20 ± 0.23	16 ± 4	2 ± 1		6 ± 2	
20	0.99 ± 0.19	92 ± 1	1.41 ± 0.07	60 ± 2	44 ± 1		30 ± 3	
28	0.37 ± 0.06	82 ± 2	3.00 ± 0.18	37 ± 2	7 ± 2		4 ± 2	
31	1.50 ± 0.13	74 ± 1	1.50 ± 0.07	46 ± 1	13 ± 2		28 ± 2	
41	1.29 ± 0.17	39 ± 1		44 ± 1	24 ± 1		35 ± 4	
42	0.18 ± 0.08	94 ± 1	0.6 ± 0.02	27 ± 4	65 ± 1	7.3 ± 1.3	69 ± 1	6.6 ± 1.2
44	0.72 ± 0.06	76 ± 1	13.9 ± 1.6	33 ± 3	74 ± 2	3.0 ± 0.4	77 ± 1	4.4 ± 0.7

[a]BRL50481: IC_{50} (PDE7A) = 0.09 μM; 0.43% inh @10 μM (PDE3A); IC_{50} (PDE4B2) = 1.80 μM; IC_{50} (PDE4D3) = 1.28 μM. [b]Rolipram: 3.10% inh @10 μM (PDE7A); 9.42% inh @10 μM (PDE3A); IC_{50} (PDE4B2) = 97.9 nM; IC_{50} (PDE4D3) = 82.5 nM. [c]Values represent the mean ±standard deviation of two independent experiments (n = 2) with duplicate measurements.

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cultured glial cells (astrocytes and microglia). Cultures were incubated with 10 μM of compounds **10**, **12**, **20**, **28**, **31**, **41**, **42**, and **44** for 1 h, and then cells were treated with LPS for a further 24 h. When primary astrocytes and microglial cells were stimulated with LPS (Figure 7), we observed a significant induction of nitrite production (approximately 5-fold) in the culture medium, which was significantly decreased after the treatment with most of the selected PDE7A inhibitors.

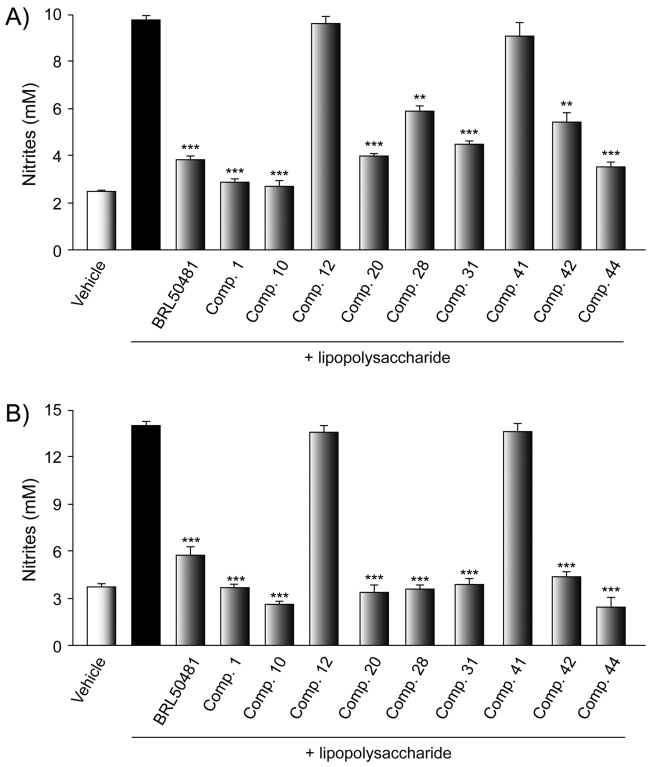

Figure 7. Effect of PDE7 inhibitors on the inflammatory response of astrocyte cultures (A) and microglial cells (B). Rat primary astrocyte or microglial cultures were treated for 24 h with LPS (10 μg/mL) in the absence or presence of PDE7 inhibitors (10 μM) and the production of nitrite was evaluated by the Griess reaction. Values represent the means ± SD from three different experiments. ***$p \leq 0.001$, **$p \leq 0.01$ versus LPS-treated cells.

Prediction of Blood–Brain Barrier Permeation. The development of a new drug candidate for a neurological disease is based not only on a good activity on the selected target but also on the drug-like properties of the candidate. To target the CNS system, the assessment of their ability to penetrate into the brain is of utmost importance.

To determine whether these compounds have a suitable drug profile to be administered in vivo, we determined their ability to cross the blood–brain barrier (BBB). The majority of compounds enter the brain by transcellular passive diffusion, which is driven by a concentration gradient between the blood and the brain.[21] The parallel artificial membrane permeability assay (PAMPA) is a high-throughput technique developed to predict passive permeability through biological membranes. Here, we used the PAMPA-BBB method described by Di et al.[22] that employs a brain lipid porcine membrane to determine the ability of new inhibitors to penetrate into the brain. The in vitro permeability (*Pe*) of commercial drugs through lipid membrane extract together with the new PDE7A inhibitors was determined (Table S2 in Supporting Information). A good correlation between the experimental and described permeability values in the literature (*Pe* (exp) = 1.8979 (lit) − 1.667 (R^2 = 0.9506)) was obtained (see Figure S1 in Supporting Information). Following the pattern established in the literature for BBB permeation prediction[23] that classify compounds as CNS+ when they present a permeability >5.92 × 10^{-6} cm s^{-1}, we can consider that all the tested sulfide-like PDE7A inhibitors (**10**, **12**, **20**, **28**, **41**, **42**, and **44**) are able to cross the BBB by passive permeation, and thus they may be used as drug candidates for CNS diseases (Table S2 in Supporting Information).

On the basis of the above-reported results, we chose sulfide **28** for further in vivo studies because of its potency and selective PDE profile and its significant efficacy in both cellular models and its ability to penetrate the brain.

Emetogenic Study of PDE7 Inhibitors. The results found in cell cultures for the sulfide-like PDE7A inhibitors confirm the biological relevance of PDE7 as a valid target for PD. Considering the great potential therapeutic use of this class of compounds, it is necessary to pharmaceutically assess their safety.

Until now, the severe gastrointestinal side effects found in the human clinical development of many different PDE4 inhibitors have ruled out the compounds from entering the market. Only roflumilast has been approved for the treatment of EPOC, both by the FDA and EMA, with a narrow therapeutic window. Emesis is a mechanism-based toxicity associated with PDE4D inhibition in humans,[24] which is not easily reproduced in preclinical models. The most common and expensive model is the use of ferrets. However, recently, some surrogate models have been developed to determine human emetogenic effects in early development stages. In fact, it was previously demonstrated that assessing the anesthesia-reversing effects of PDE4 inhibitors is a novel surrogate to evaluate the emetic potential of these compounds in animal species that do not have a vomiting reflex, e.g., mice.[19] In the presence of PDE4 inhibitors, the percentage of mice exhibiting loss of the righting reflex was reduced and the duration of anesthesia was shortened.[15]

Three different compounds were used as respective controls: (*R*)-rolipram (1 mg/kg, sc), roflumilast (1 mg/kg, sc), and BRL50481 (5 mg/kg, sc). Rolipram and roflumilast are reference inhibitors of PDE4, exhibiting emetic-related effects,[19,25] whereas BRL50481[20] is a known inhibitor of PDE7 in which the emetic effects have not been shown. The doses of each reference compound were selected in preliminary experiments performed in our laboratory (detailed in the Experimental Section). The return of the righting reflex (when the mouse that was on its back turned itself spontaneously to a prone position) was used as an end point to measure the duration of anesthesia.

In our experimental conditions, the combined administration of xylacine (10 mg/kg) and ketamine (100 mg/kg) induced loss of the righting reflex within 15 min following injection. Individual group comparisons showed that the administration of rolipram (1 mg/kg) and roflumilast (1 mg/kg) induced a decrease in the duration of anesthesia (both $p < 0.05$), but the administration of the PDE7 inhibitor BRL50481 (5 mg/kg), using a 5-fold greater dose, did not induce any effect. Moreover, the sulfide-like PDE7A inhibitor **28** at the doses evaluated (2.5, 5, and 10 mg/kg) was not able to modify the duration of anesthesia induced by the combination of ketamine and xylacine (Figure 8), supporting the absence of emetic-related

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effects in PDE7 inhibitors in general and in the sulfide compound **28** in particular.

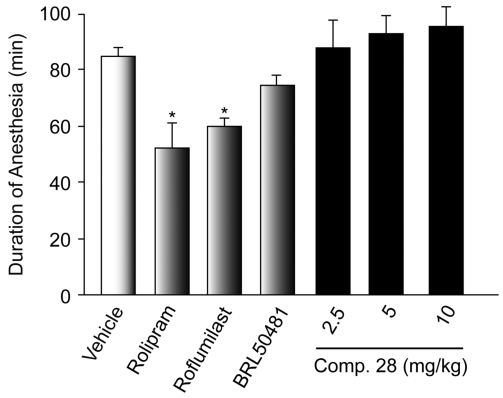

Figure 8. Effects of compound **28** at different concentrations (2.5, 5, and 10 mg/kg) on the duration of anesthesia induced by the combination of xylazine (10 mg/kg) and ketamine (100 mg/kg) in CD1 mice. $F(6, 107) = 4.617$, $p < 0.01$. *$p < 0.05$ versus vehicle. The evaluated compounds were injected subcutaneously (sc).

To confirm the lack of emetic effects when PDE7 is inhibited, we evaluated the anesthesia-reversing behavior of different groups of mice exposed to the presence of three chemically diverse PDE7 inhibitors previously synthesized in our group. Currently, there are two approaches for studying the function of a specific protein: classical genetics (using antibodies, mutations, siRNAs, etc.) or chemical genetics (using small chemical probes that target this protein). This is the approach used here to show the lack of emetogenic activity when PDE7 is down-regulated. Thus, a quinazoline derivative (3-phenyl-2,4-dithioxo-1,2,3,4-tetrahydroquinazoline (TC3.6), IC_{50} = 1.04 μM),[26] a furan derivative (3,4,5-trimethoxybenzyl 5-phenyl-2-furoate (MR1.51), IC_{50} = 5.17 μM),[27] and an iminothiadiazole (5-(2-hydroxyethylimino)-2,3-diphenyl-2,5-dihydro-1,2,4-thiadiazole hydrobromide (VP1.15), IC_{50} = 1.11 μM)[28] were used in this surrogate in vivo model. As expected, none of them induced any effect in the animals assayed, confirming the lack of emetogenic activity after the use of cAMP-specific PDE7 inhibitors (Figures 8 and 9).

CONCLUSIONS

We corroborated the great potential of PDE7A inhibitors as a new therapeutic option for neurodegenerative diseases, specifically Parkinson's disease. Moreover, we have shown that PDE7 inhibitors may be a real therapeutic option for human pharmacological treatments, as they do not have the emetogenic activity found in clinical trials of PDE4 inhibitors. PDE7 inhibitors are promising drug candidates for pathologies in which cAMP signaling is involved such as the inflammatory process of the central nervous system.

Here, we have shown that chemical genetic approaches provide innovative hits that can be fine-tuned by medicinal chemistry programs to obtain valuable drug candidates. We have shown here that sulfide-like PDE7A inhibitors are effective compounds in cellular models for Parkinson's disease, they are brain-penetrating drugs and these PDE7A inhibitors have no emetic effects in a surrogate animal model. Overall, PDE7A inhibitors represent good drug candidates for CNS diseases, especially Parkinson's disease, as they are free of potential emetic side effects.

EXPERIMENTAL SECTION

Chemical Procedures. Substrates were purchased from commercial sources and used without further purification. Melting points were determined with a Mettler Toledo MP70 apparatus. Flash column chromatography was carried out at medium pressure using silica gel (E. Merck, grade 60, particle size 0.040–0.063 mm, 230–240 mesh ASTM) with the indicated solvent as eluent. Compounds were detected with UV light (254 nm). ^{1}H NMR spectra were obtained on the Bruker AVANCE-300 spectrometer working at 300 MHz or on a Varian INOVA 400 spectrometer working at 400 MHz. Typical spectral parameters: spectral width 10 ppm, pulse width 9 μs (57°), data size 32 K. ^{13}C NMR experiments were carried out on the Bruker AVANCE-300 spectrometer operating at 75 MHz or on a Varian INOVA 400 spectrometer working at 100 MHz. The acquisition parameters: spectral width 16 kHz, acquisition time 0.99 s, pulse width

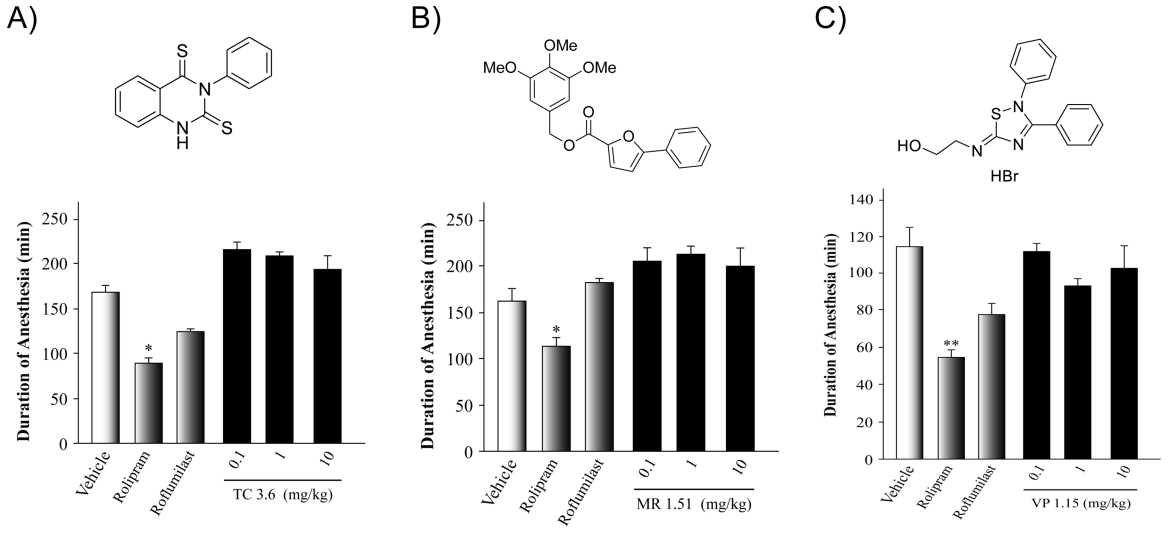

Figure 9. Effects of chemically diverse PDE7 inhibitors on the duration of anesthesia induced by the combination of xylazine (10 mg/kg) and ketamine (80 mg/kg) in CD1 mice. (A) Compound tested: TC3.6, $F(5, 45) = 12.003$, $p < 0.01$. *$p < 0.05$ versus vehicle. (B) Compound tested MR1.51, $F(5, 58) = 9.776$, $p < 0.001$. *$p < 0.05$ versus vehicle. (C) Compound tested: VP1.15, $F(5, 56) = 5.508$, $p < 0.01$. **$p < 0.01$ versus vehicle. Rolipram and roflumilast, both PDE4 inhibitors, have been used as positive controls. The evaluated compounds were injected subcutaneously (sc).

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9 μs (57°), data size 32 K. Chemical shifts are reported in values (ppm) relative to internal Me_4Si, and J values are reported in Hz. HPLC analyses were performed on Alliance Waters 2690 equipment, with a UV detector photodiode array Waters 2996 with MS detector MicromassZQ (Waters), using an Sunfire column C18, 3.5 μm (50 mm × 4.6 mm) and acetonitrile and Milli-Q water (with 0.1% formic acid) as mobile phase. The standard gradient consisted of a 5 min run from 15% to 95% of acetonitrile at a flow rate of 1 mL/min. Elemental analysis results of all the new compounds were recorded on Heraeus CHN-O rapid analyzer performed by the analytical department at CENQUIOR (CSIC) and values were within ±0.4% of the theoretical values for all compounds; therefore, these compounds meet the criteria of ≥95%. Additionally, purity of all final compounds was found to be ≥95% by LC/MS analysis. The microwave assisted syntheses were carried out using a Biotage Initiator 8 single-mode cavity instrument from Biotage. Experiments were performed with temperature control mode in sealed microwave process vials. The temperature was measured with an IR sensor on the outside of the reaction vessel. Stirring was provided by an in situ magnetic stirrer.

General Synthetic Procedure for Compounds 3−45 and 55−58. A solution of aryl thiol and aryl halide in the corresponding solvent was prepared. The base was added, and the mixture was heated under microwave irradiation during the time and temperature indicated in each case. After cooling down, the solvent was evaporated and the crude was washed with a saturated NaCl solution. The organic phase was dried over magnesium sulfate and chromatographed on silica gel column using as eluents mixtures of solvents in the portions indicated.

*2-(4,5-Dichloro-2-nitrophenylthio)aniline (**3**).* Reagents: 2-aminothiophenol (1.2 mmol, 1 equiv, 150.0 mg), 1,2-dichloro-4-fluoro-5-nitrobenzene (1.4 mmol, 1.2 equiv, 300.4 mg), and potassium carbonate (6.0 mmol, 5 equiv, 826.7 mg). Solvent: anhydrous dichloromethane. Microwave irradiation (80 °C, 1 h 30 min). Purification: ethyl acetate:hexane (1:4). Yield: orange solid (196.0 mg, 52%); mp 158−159 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.51 (s, 1H), 7.35 (dd, J = 7.7, 1.6 Hz, 1H), 7.29 (ddd, J = 8.1, 7.2, 1.6 Hz, 1H), 6.84 (dd, J = 8.1, 1.2 Hz, 1H), 6.74 (s, 1H), 6.65 (ddd, J = 7.7, 7.2, 1.2 Hz, 1H), 5.65 (s, 2H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 151.6, 144.8, 136.7, 138.2, 138.1, 133.5, 128.5, 128.3, 128.2, 117.7, 116.1, 109.0. HPLC: purity 97%. MS (IE) (m/z): 315 [M], 317 [M + 2]. Anal. ($C_{12}H_8Cl_2N_2O_2S$) C, H, N, S.

*2-[2-(Trifluoromethyl)-4-nitrophenylthio]aniline (**4**).* Reagents: 2-aminothiophenol (1.2 mmol, 1 equiv, 150.0 mg), 1-chloro-4-nitro-2-(trifluoromethyl)benzene (1.4 mmol, 1.2 equiv, 324.3 mg), and potassium carbonate (6.0 mmol, 5 equiv, 826.7 mg). Solvent: anhydrous dichloromethane. Microwave irradiation (80 °C, 1h 30 min). Purification: ethyl acetate:hexane (1:4). Yield: orange solid (243.8 mg, 65%); mp 105−106 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.41 (d, J = 2.5 Hz, 1H), 8.33 (dd, J = 8.8, 2.5, Hz, 1H), 7.33 (dd, J = 7.4, 1.5 Hz, 1H), 7.30 (ddd, J = 8.0, 7.4, 1.5 Hz, 1H), 6.94 (dd, J = 8.8 Hz, 1H), 6.90 (dd, J = 8.0, 1.3 Hz, 1H), 6.65 (td, J = 7.4, 1.3 Hz, 1H), 5.57 (s, 2H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 152.0, 147.3, 145.2, 138.3, 133.6, 128.4, 128.1, 126.1 (q, J = 32.4 Hz), 123.8 (q, J = 274.3 Hz, CF_3), 123.0 (q, J = 5.9 Hz), 118.2, 116.5, 107.9. HPLC: purity >99%. MS (ESI+) (m/z): 315 [M + 1]. Anal. ($C_{13}H_9F_3N_2O_2S$) C, H, N, S.

*2-(2-Chloro-4-nitrophenylthio)aniline (**5**).* Reagents: 2-aminothiophenol (1.2 mmol, 1 equiv, 150.0 mg), 2-chloro-1-fluoro-4-nitrobenzene (1.4 mmol, 1.2 equiv, 252.4 mg), and potassium carbonate (6.0 mmol, 5 equiv, 826.7 mg). Solvent: anhydrous dichloromethane. Microwave irradiation (80 °C, 1h 30 min). Purification: ethyl acetate:hexane (1:4). Yield: orange solid (133.7 mg, 40%); mp 128−129 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.24 (d, J = 2.3 Hz, 1H), 7.90 (dd, J = 8.9, 2.3 Hz, 1H), 7.44 (ddd, J = 7.8, 1.6, 0.5 Hz, 1H), 7.36 (ddd, J = 8.2, 7.3, 1.6 Hz, 1H), 6.94−6.77 (m, 2H), 6.71 (d, J = 8.9 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 149.7, 146.5, 145.7, 138.1, 133.1, 131.2, 126.1, 125.0, 122.4, 119.8, 116.2, 110.7. HPLC: purity 97%. MS (ESI+) (m/z): 281 [M + 1], 283 [M + 3]. Anal. ($C_{12}H_9ClN_2O_2S$) C, H, N, S.

*2-(4-Fluoro-2-nitrophenylthio)aniline (**6**).* Reagents: 2-aminothiophenol (1.19 mmol, 1 equiv, 150.0 mg), 1,4-difluoro-2-nitrobenzene (1.4 mmol, 1.2 equiv, 228.8 mg), and potassium carbonate (6.0 mmol, 5 equiv, 826.7 mg). Microwave irradiation (80 °C, 1 h 30 min). Solvent: anhydrous dichloromethane. Microwave irradiation (80 °C, 1 h 30 min). Purification: ethyl acetate:hexane (1:9). Yield: orange solid (162.1 mg, 51%); mp 71−72 °C (lit.[29] 73−74 °C). ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.14 (dd, J = 8.8, 2.8 Hz, 1H), 7.53 (ddd, J = 9.1, 7.8, 2.9 Hz, 1H), 7.34 (dd, J = 7.7, 1.6 Hz, 1H), 7.25 (ddd, J = 8.2, 7.2, 1.6 Hz, 1H), 6.81 (ddd, J = 8.1, 1.4, 0.4 Hz, 1H), 6.76 (dd, J = 9.1, 5.3 Hz, 1H), 6.63 (ddd, J = 7.7, 7.2, 1.4 Hz, 1H), 5.56 (s, 2H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 159.5 (d, J = 248.8 Hz), 148.6, 145.3 (d, J = 7.9 Hz), 137.9, 132.8, 132.5, 128.9 (d, J = 7.3 Hz), 121.7 (d, J = 21.9 Hz), 119.9, 116.2, 113.1 (d, J = 26.7 Hz), 112.7. HPLC: purity 97%. MS (ESI+) (m/z): 265 [M +1]. Anal. ($C_{12}H_9FN_2O_2S$) C, H, N, S.

*4-[2-(Trifluoromethyl)-4-nitrophenylthio]aniline (**7**).* Reagents: 4-aminothiophenol (1.2 mmol, 1 equiv, 150.0 mg), 1-chloro-4-nitro-2-(trifluoromethyl)benzene (1.4 mmol, 1.2 equiv, 324.3 mg), and potassium carbonate (6.0 mmol, 5 equiv, 826.7 mg). Solvent: anhydrous dichloromethane. Microwave irradiation (100 °C, 45 min). Purification: ethyl acetate:hexane (1:9). Yield: orange solid (197.0 mg, 53%); mp 95−96 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.37 (d, J = 2.5 Hz, 1H), 8.30 (dd, J = 8.8, 2.5 Hz, 1H), 7.23 (d, J = 8.5 Hz, 2H), 7.01 (d, J = 8.8 Hz, 1H), 6.70 (d, J = 8.5 Hz, 2H), 5.83 (s, 2H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 150.8, 148.6, 144.0, 137.7, 127.7, 126.3 (q, J = 32.4 Hz), 122.7 (q, J = 274.6 Hz), 122.2 (q, J = 6.0 Hz), 116.4, 115.1. HPLC: purity 97%. MS (ESI+) (m/z): 315 [M + 1]. Anal. ($C_{13}H_9F_3N_2O_2S$) C, H, N, S.

*4-(2-Chloro-4-nitrophenylthio)aniline (**8**).* Reagents: 4-aminothiophenol (0.8 mmol, 1 equiv, 100.0 mg), 2-chloro-1-fluoro-4-nitrobenzene (1.0 mmol, 1.2 equiv, 168.3 mg), and potassium carbonate (4.0 mmol, 5 equiv, 551.3 mg). Solvent: anhydrous dichloromethane. Microwave irradiation (100 °C, 45 min). Purification: ethyl acetate:hexane (1:4). Yield: orange solid (202.0 mg, 45%); mp 138−139 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.26 (d, J = 2.6 Hz), 8.05 (dd, J = 8.9, 2.6 Hz, 1H), 7.22 (d, J = 8.5 Hz, 2H), 6.75 (d, J = 8.9 Hz, 1H), 6.70 (d, J = 8.5 Hz, 2H), 5.79 (s, 2H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 151.0, 149.7, 144.24, 136.8, 127.9, 125.4, 123.8, 122.3, 115.0, 109.4. HPLC: purity >99%. MS (ESI+) (m/z): 281 [M + 1], 283 [M + 3]. Anal. ($C_{13}H_9ClN_2O_2S$) C, H, N, S.

*4-(5-Chloro-2-nitrophenylthio)aniline (**9**).* Reagents: 4-aminothiophenol (1.6 mmol, 1 equiv, 200.0 mg), 2,4-dichloro-1-nitrobenzene (1.9 mmol, 1.2 equiv, 368.1 mg), and potassium carbonate (8.0 mmol, 5 equiv, 1.1 g). Solvent: anhydrous dichloromethane. Microwave irradiation (100 °C, 45 min). Purification: ethyl acetate:hexane (1:4). Isomers mixture were obtained and separated by semipreparative HPLC using as eluents methanol:acetonitrile 3:4. Yield: yellow solid (34.1 mg, 77%); mp 113−114 °C (lit[30] 109−111 °C). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.22 (d, J = 8.8 Hz, 1H), 7.36 (dd, J = 8.8, 2.2 Hz, 1H), 7.21 (d, J = 8.5 Hz, 2H), 6.69 (d, J = 8.5 Hz, 2H), 6.66 (d, J = 2.2 Hz, 1H), 5.76 (s, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 152.0, 144.2, 143.2, 140.2, 137.9, 128.5, 126.8, 125.9, 116.0, 112.1. HPLC: purity >99%. MS (ESI+) (m/z): 281 [M + 1], 283 [M + 3]. Anal. ($C_{13}H_9ClN_2O_2S$) C, H, N, S.

*4-(3-Chloro-4-nitrophenylthio)aniline (**10**).* Reagents: 4-aminothiophenol (1.6 mmol, 1 equiv, 200.0 mg), 2,4-dichloro-1-nitrobenzene (1.9 mmol, 1.2 equiv, 368.1 mg), and potassium carbonate (8.0 mmol, 5 equiv, 1103.0 mg). Solvent: anhydrous dichloromethane. Microwave irradiation (100 °C, 45 min). Purification: ethyl acetate:hexane (1:4). Isomers mixture were obtained. These isomers were separated by semipreparative HPLC using as eluents methanol:acetonitrile 3:4. Yield: yellow solid (15.8 mg, 61%); mp 129−130 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.94 (d, J = 8.7 Hz, 1H), 7.19 (d, J = 8.5 Hz, 2H), 7.12 (d, J = 2.0 Hz, 1H), 7.03 (dd, J = 8.7, 2.0 Hz, 1H), 6.65 (d, J = 8.5 Hz, 2H), 5.72 (s, 2H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 151.8, 151.0, 144.0, 137.7, 127.5, 127.2, 126.9, 124.5, 115.8, 111.3. HPLC: purity >99%. MS (ESI+) (m/z): 281 [M + 1], 283 [M + 3]. Anal. ($C_{13}H_9ClN_2O_2S$) C, H, N, S.

*4-Bromophenyl 5-Chloro-2-nitrophenyl sulfide (**11**).* Reagents: 4-bromothiophenol (1.1 mmol, 1 equiv, 200.0 mg), 2,4-dichoro-1-nitrobenzene (1.3 mmol, 1.2 equiv, 243.7 mg), and potassium carbonate (5.3 mmol, 5 equiv, 730.0 mg). Solvent: anhydrous

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dichloromethane. Microwave irradiation (80 °C, 1 h 30 min). Purification: ethyl acetate:hexane (1:4). Yield: yellow solid (114.4 mg, 32%); mp 131−132 °C (lit[31] 127.7−128.6 °C). ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.27 (d, J = 8.9 Hz, 1H), 7.78 (d, J = 8.5 Hz, 2H), 7.59 (d, J = 8.5 Hz, 2H), 7.49 (dd, J = 8.9, 2.2 Hz, 1H), 6.74 (d, J = 2.2 Hz, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 143.5, 139.5, 139.4, 137.4, 133.6, 129.1, 127.9, 127.1, 126.3, 124.6. HPLC: purity 97%. MS (IE) (m/z): 344 [M], 346 [M + 2]. Anal. ($C_{12}H_7BrClNO_2S$) C, H, N, S.

*2-Bromophenyl 5-Chloro-2-nitrophenyl Sulfide (**12**).* Reagents: 2-bromothiophenol (0.8 mmol, 1 equiv, 150.0 mg), 2,5-dichloronitrobenzene (1.0 mmol, 1.2 equiv, 182.8 mg), and potassium carbonate (4.0 mmol, 5 equiv, 547.2 mg). Solvent: anhydrous dichloromethane. Microwave irradiation (80 °C, 1 h 30 min). Purification: ethyl acetate:hexane (1:9). Yield: yellow solid (43.0 mg, 16%); mp 100−101 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.32 (d, J = 8.9 Hz, 1H), 7.93 (dd, J = 7.6, 1.8 Hz, 1H), 7.85 (dd, J = 7.6, 1.9 Hz, 1H), 7.58 (dd, J = 7.6, 1.8 Hz, 1H), 7.55 (dd, J = 7.6, 1.9 Hz, 1H), 6.57 (d, J = 2.0 Hz, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 143.5, 139.7, 138.2, 138.1, 134.5, 133.1, 130.4, 129.9, 128.2, 128.1, 126.5 (2C). HPLC: purity 97%. MS (EI) (m/z): 344 [M], 346 [M + 2]. Anal. ($C_{12}H_7BrClNO_2S$) C, H, N, S.

*2-Bromophenyl 2-Chloro-4-nitrophenyl sulfide (**13**).* Reagents: 2-bromothiophenol (0.8 mmol, 1 equiv, 150.0 mg), 3-chloro-4-fluoronitrobenzene (1.0 mmol, 1.2 equiv, 166.8 mg), and potassium carbonate (4.0 mmol, 5 equiv, 547.2 mg). Solvent: anhydrous dichloromethane. Microwave irradiation (80 °C, 1 h 30 min). Purification: ethyl acetate:hexane (1:9). Yield: yellow solid (120.0 mg, 40%); mp 91−92 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.36 (d, J = 2.5 Hz, 1H), 8.09 (dd, J = 8.8, 2.5 Hz, 1H), 7.93 (dd, J = 7.0, 2.3 Hz, 1H), 7.76 (dd, J = 7.0, 2.3 Hz, 1H), 7.61−7.49 (m, 2H), 6.79 (d, J = 8.8 Hz, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 145.6, 144.5, 137.4, 134.4, 132.7, 130.0, 129.7, 129.4, 129.3, 127.2, 124.6, 123.0. HPLC: purity 97%. MS (IE) (m/z): 344 [M], 346 [M + 2]. Anal. ($C_{12}H_7BrClNO_2S$) C, H, N, S.

*2-(5-Chloro-2-nitrophenylthio)aniline (**14**).* Reagents: 2-aminothiophenol (1.6 mmol, 1 equiv, 200.0 mg), 2,4-dichloro-1-nitrobenzene (1.6 mmol, 1 equiv, 306.8 mg), and sodium acetate (2.4 mmol, 1 equiv, 232.5 mg). Solvent: ethanol. Microwave irradiation (85 °C, 2 h). Purification: ethyl acetate:hexane (1:9). Yield: orange solid (224.0 mg, 50%); mp 134−135 °C (lit.[32] 132.0−133.5 °C). ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.28 (d, J = 8.8 Hz, 1H), 7.42 (dd, J = 8.8, 2.2 Hz, 1H), 7.34 (dd, J = 7.5, 1.6 Hz, 1H), 7.28 (ddd, J = 8.2, 7.5, 1.6 Hz, 1H), 6.84 (dd, J = 8.2, 1.3 Hz, 1H), 6.67 (dd, J = 7.5, 1.3 Hz, 1H), 6.63 (d, J = 2.2 Hz, 1H), 5.63 (s, 2H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 150.8, 143.7, 139.5, 139.2, 137.3, 132.5, 128.0, 125.6, 125.3, 116.9, 115.2, 108.6. HPLC: purity >99%. MS (ESI+) (m/z): 281 [M + 1], 283 [M + 3]. Anal. ($C_{12}H_9ClN_2O_2S$) C, H, N, S.

*2-(4-Chloro-2-nitrophenylthio)aniline (**15**).* Reagents: 2-aminothiophenol (1.6 mmol, 1 equiv, 200.0 mg), 2,5-dichloronitrobenzene (1.6 mmol, 1 equiv, 306.8 mg), and sodium acetate (2.4 mmol, 1.5 equiv, 232.5 mg). Solvent: ethanol. Microwave irradiation (85 °C, 2 h). Purification: ethyl acetate:hexane (3:17). Yield: yellow solid (268.1 mg, 60%); mp 121−122 °C (lit.[33] 122.0 °C). ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.28 (d, J = 2.1 Hz, 1H), 7.65 (dd, J = 8.8, 2.1 Hz, 1H), 7.34 (dd, J = 7.7, 1.6 Hz, 1H), 7.25 (ddd, J = 8.2, 7.2, 1.6 Hz, 1H), 6.81 (dd, J = 8.2, 1.3 Hz, 1H), 6.74 (d, J = 8.8 Hz, 1H), 6.63 (ddd, J = 7.7, 7.2, 1.3 Hz, 1H), 5.57 (s, 2H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 150.8, 145.4, 137.3, 136.1, 133.8, 132.3, 129.3, 128.4, 125.4, 116.7, 115.1, 108.8. HPLC: purity >99%. MS (ESI+) (m/z): 281 [M + 1], 283 [M + 3]. Anal. ($C_{12}H_9ClN_2O_2S$) C, H, N, S.

*3-(4-Chloro-2-nitrophenylthio)aniline (**16**).* Reagents: 3-aminothiophenol (1.2 mmol, 1 equiv, 150.0 mg), 2,5-dichloronitrobenzene (1.2 mmol, 1 equiv, 230.4 mg), and sodium acetate (1.8 mmol, 1.5 equiv, 174.6 mg). Solvent: ethanol. Microwave irradiation (85 °C, 2 h). Purification: ethyl acetate:hexane (1:9). Yield: orange solid (124.8 mg, 32%); mp 96−97 °C (lit.[34] 94.0 °C). ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.24 (d, J = 2.4 Hz, 1H), 7.66 (dd, J = 8.8, 2.4 Hz, 1H), 7.15 (t, J = 7.8 Hz, 1H), 6.92 (d, J = 8.8 Hz, 1H), 6.72 (t, J = 2.0 Hz, 1H), 6.73−6.65 (m, 2H), 5.44 (s, 2H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 151.2, 145.6, 137.9, 134.8, 131.6, 130.4, 130.4, 130.1, 125.8, 122.4, 120.1, 116.4. HPLC: purity >99%. MS (ESI+) (m/z): 281 [M + 1], 283 [M + 3]. Anal. ($C_{12}H_9ClN_2O_2S$) C, H, N, S.

*3-(2-Chloro-4-nitrophenylthio)aniline (**17**).* Reagents: 3-aminothiophenol (1.6 mmol, 1 equiv, 200.0 mg), 3-chloro-4-fluoronitrobenzene (1.6 mmol, 1 equiv, 280.8 mg), and sodium acetate (2.4 mmol, 1.5 equiv, 232.8 mg). Solvent: ethanol. Microwave irradiation (85 °C, 2 h). Purification: ethyl acetate:hexane (1:4). Yield: orange solid (344.3 mg, 77%); mp 123−124 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.29 (d, J = 2.4 Hz, 1H), 8.08 (dd, J = 8.9, 2.4 Hz, 1H), 7.20 (t, J = 7.7 Hz, 1H), 6.90 (d, J = 8.9 Hz, 1H), 6.76 (t, J = 1.9 Hz, 1H), 6.75−6.69 (m, 2H), 5.49 (s, 2H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 150.6, 147.8, 144.9, 131.0, 129.0, 127.7, 126.5, 124.2, 122.8, 121.7, 119.5, 115.9. HPLC: purity >99%. MS (ESI+) (m/z): 281 [M + 1], 283 [M + 3]. Anal. ($C_{12}H_9ClN_2O_2S$) C, H, N, S.

*4-(4-Fluoro-2-nitrophenylthio)aniline (**18**).* Reagents: 4-aminothiophenol (2.0 mmol, 1 equiv, 250.0 mg), 1,4-difluoro-2-nitrobenzene (2.0 mmol, 1 equiv, 317.7 mg), and sodium acetate (1.2 mmol, 1.5 equiv, 115.4 mg). Solvent: ethanol. Microwave irradiation (85 °C, 1h 30 min). Purification: ethyl acetate:hexane (1:4). Yield: orange solid (283.6 mg, 54%); mp 124−125 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.11 (dd, J = 8.7, 2.9 Hz, 1H), 7.54 (ddd, J = 9.0, 7.8, 2.9 Hz, 1H), 7.22 (d, J = 8.5 Hz, 2H), 6.87 (dd, J = 9.0, 5.3 Hz, 1H), 6.69 (d, J = 8.5 Hz, 2H), 5.74 (s, 2H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 151.5, 141.8 (d, J = 376 Hz), 137.6, 136.9 (d, J = 11.7 Hz), 129.8, 129.7 (d, J = 7.8 Hz), 122.5 (d, J = 22.4 Hz), 115.6, 112.9, 112.5. HPLC: purity 97%. MS (ESI+) (m/z): 265 [M + 1]. Anal. ($C_{12}H_9FN_2O_2S$) C, H, N, S.

*2-Bromophenyl 4,5-Dichloro-2-nitrophenyl Sulfide (**19**).* Reagents: 2-bromothiophenol (0.8 mmol, 1 equiv, 150.0 mg), 1,2-dichloro-4-fluoro-5-nitrobenzene (0.8 mmol, 1 equiv, 166.5 mg), and sodium acetate (1.2 mmol, 1.5 equiv, 115.4 mg). Solvent: ethanol. Microwave irradiation (85 °C, 1h 30 min). Purification: ethyl acetate:hexane (1:10). Yield: yellow solid (49.2 mg, 17%); mp 156−157 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.57 (s, 1H), 7.93 (dd, J = 7.2, 1.2 Hz, 1H), 7.83 (dd, J = 7.2, 1.6 Hz, 1H), 7.68−7.42 (m, 2H), 6.78 (s, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 143.9, 137.8, 137.7, 136.0, 134.4, 133.0, 130.2, 129.8, 129.4, 128.8, 128.5, 127.8. HPLC: purity 97%. MS (IE) (m/z): 379 [M], 381 [M + 2]. Anal. ($C_{12}H_6BrCl_2NO_2S$) C, H, N, S.

*2-Bromophenyl 4-chloro-2-nitrophenyl sulfide (**20**).* Reagents: 2-bromothiophenol (0.8 mmol, 1 equiv, 150.0 mg), 2,5-dichloronitrobenzene (0.8 mmol, 1 equiv, 152.3 mg), and sodium acetate (1.2 mmol, 1.5 equiv, 115.4 mg). Solvent: ethanol. Microwave irradiation (85 °C, 1 h 30 min). Purification: ethyl acetate:hexane (1:9). Yield: yellow solid (199.4 mg, 70%); mp 85−86 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.33 (d, J = 2.4 Hz, 1H), 7.89 (dd, J = 7.4, 1.8 Hz, 1H), 7.80 (dd, J = 7.4, 2.0 Hz, 1H), 7.70 (dd, J = 8.8, 2.4 Hz, 1H), 7.56 (dd, J = 7.4, 1.8 Hz, 1H), 7.51 (dd, J = 7.4, 2.0 Hz, 1H), 6.78 (d, J = 8.8 Hz, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 145.5, 137.7, 134.4, 134.3, 134.2, 132.6, 130.8, 130.6, 129.7, 129.6, 129.5, 125.5. HPLC: purity 97%. MS (IE) (m/z): 344 [M], 346 [M + 2]. Anal. ($C_{12}H_7BrClNO_2S$) C, H, N, S.

*4-Bromophenyl 2-Chloro-4-nitrophenyl Sulfide (**21**).* Reagents: 4-bromothiophenol (1.1 mmol, 1 equiv, 200.0 mg), 3-chloro-4-fluoronitrobenzene (1.1 mmol, 1 equiv, 185.7 mg), and sodium acetate (1.6 mmol, 1.5 equiv, 153.9 mg). Solvent: ethanol. Microwave irradiation (85 °C, 1h 30 min). Purification: ethyl acetate:hexane (1:9). Yield: yellow solid (216.4 mg, 57%); mp 127−128 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.34 (d, J = 2.5 Hz, 1H), 8.06 (dd, J = 8.9, 2.5 Hz, 1H), 7.78 (d, J = 8.5 Hz, 2H), 7.58 (d, J = 8.5 Hz, 2H), 6.90 (d, J = 8.9 Hz, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 146.1, 145.3, 137.1, 133.6, 129.6, 127.7, 127.2, 124.4, 124.4, 122.9. HPLC: purity >99%. MS (IE) (m/z): 344 [M], 346 [M + 2]. Anal. ($C_{12}H_7BrClNO_2S$) C, H, N, S.

*4-Bromophenyl 4-Chloro-2-nitrophenyl Sulfide (**22**).* Reagents: 4-bromothiophenol (1.1 mmol, 1 equiv, 200.0 mg), 3-chloro-4-fluoronitrobenzene (1.1 mmol, 1 equiv, 203.1 mg), and sodium acetate (1.6 mmol, 1.5 equiv, 153.9 mg). Solvent: ethanol. Microwave irradiation (85 °C, 1h 30 min). Purification: ethyl acetate:hexane (1:9). Yield: yellow solid (310.0 mg, 85%); mp 155−156 °C (lit.[35] 155

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°C). ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.29 (d, J = 2.4 Hz, 1H), 7.74 (d, J = 8.7 Hz, 2H), 7.67 (dd, J = 8.8, 2.4 Hz, 1H), 7.55 (d, J = 8.7 Hz, 2H), 6.91 (d, J = 8.8 Hz, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 145.9, 137.5, 135.9, 134.6, 133.8, 130.8, 130.6, 129.9, 125.7, 124.6. HPLC: purity >99%. MS (IE) (m/z): 344 [M], 346 [M + 2]. Anal. ($C_{12}H_7BrClNO_2S$) C, H, N, S.

*2-Bromobenzyl 2-Chloro-4-nitrophenyl Sulfide (**23**).* Reagents: 2-bromobenzyl mercaptan (0.5 mmol, 1 equiv, 100.8 mg), 3-chloro-4-fluoronitrobenzene (0.5 mmol, 1 equiv, 87.1 mg), and sodium acetate (0.7 mmol, 1.5 equiv, 72.2 mg). Solvent: ethanol. Microwave irradiation (85 °C, 1h 30 min). Purification: ethyl acetate:hexane (1:9). Yield: yellow solid (57.0 mg, 32%); mp 107−108 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.30 (d, J = 2.4 Hz, 1H), 8.18 (dd, J = 8.8, 2.4 Hz, 1H), 7.73 (d, J = 8.8 Hz, 1H), 7.68 (dd, J = 7.9, 1.3 Hz, 1H), 7.61 (dd, J = 7.6, 1.7 Hz, 1H), 7.39 (td, J = 7.6, 1.3 Hz, 1H), 7.28 (td, J = 7.9, 1.7 Hz, 1H), 4.52 (s, 2H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 146.3, 145.1, 134.6, 133.5, 131.9, 130.5, 130.2, 128.6, 126.8, 124.6, 124.4, 123.0, 36.6. HPLC: purity >99%. MS (IE) (m/z): 358 [M], 360 [M + 2]. Anal. ($C_{13}H_9BrClNO_2S$) C, H, N, S.

*2-Bromobenzyl 4-Chloro-2-nitrophenyl Sulfide (**24**).* Reagents: 2-bromobenzyl mercaptan (0.5 mmol, 1 equiv, 100.8 mg), 2,5-dichloronitrobenzene (0.5 mmol, 1 equiv, 95.2 mg), and sodium acetate (0.7 mmol, 1.5 equiv, 72.2 mg). Solvent: ethanol. Microwave irradiation (85 °C, 1h 30 min). Purification: ethyl acetate:hexane (1:10). Yield: yellow solid (80.0 mg, 45%); mp 157−158 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.27 (d, J = 2.3 Hz, 1H), 7.83 (dd, J = 8.7, 2.3 Hz, 1H), 7.76 (d, J = 8.7 Hz, 1H), 7.67 (dd, J = 7.7, 1.3 Hz, 1H), 7.56 (dd, J = 7.7, 1.7 Hz, 1H), 7.38 (td, J = 7.7, 1.3 Hz, 1H), 7.27 (td, J = 7.7, 1.7 Hz, 1H), 4.44 (s, 2H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 146.0, 134.9, 134.4, 134.0, 133.0, 131.7, 130.0, 129.8, 129.6, 128.2, 125.4, 124.2, 37.2. HPLC: purity >99%. MS (IE) (m/z): 358 [M], 360 [M + 2]. Anal. ($C_{13}H_9BrClNO_2S$) C, H, N, S.

*2-Chloro-4-nitrophenyl 2-isopropylphenyl Sulfide (**25**).* Reagents: 2-isopropyl isopropylthiophenol (0.7 mmol, 1 equiv, 100.0 mg), 3-chloro-4-fluoronitrobenzene (0.7 mmol, 1 equiv, 115.3 mg), and sodium acetate (1.0 mmol, 1.5 equiv, 93.1 mg). Solvent: ethanol. Microwave irradiation (85 °C, 1h 30 min). Purification: ethyl acetate:hexane (1:40). Yield: brown oil (143.6 mg, 48%). ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.34 (d, J = 2.5 Hz, 1H), 8.07 (dd, J = 8.9, 2.5 Hz, 1H), 7.66−7.61 (m, 2H), 7.59 (dd, J = 7.7, 1.0 Hz, 1H), 7.43−7.39 (m, 1H), 6.64 (d, J = 8.9 Hz, 1H), 3.34 (m, 1H), 1.16 (d, J = 6.9 Hz, 6H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 153.2, 147.7, 145.3, 137.4, 132.2, 129.5, 128.4, 127.9, 126.5, 126.3, 124.7, 123.2, 31.1, 23.9. HPLC: purity 97%. MS (IE) (m/z): 307 [M], 309 [M + 2]. Anal. ($C_{15}H_{14}ClNO_2S$) C, H, N, S.

*2-Chloro-4-nitrophenyl 4-isopropylphenyl Sulfide (**26**).* Reagents: 4-isopropylthiophenol (0.7 mmol, 1 equiv, 100.0 mg), 3-chloro-4-fluoronitrobenzene (0.7 mmol, 1 equiv, 115.3 mg), and sodium acetate (1.0 mmol, 1.5 equiv, 93.1 mg). Solvent: ethanol. Microwave irradiation (85 °C, 1h 30 min). Purification: ethyl acetate:hexane (1:60). Yield: pale-yellow solid (57.4 mg, 30%); mp 71−72 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.32 (d, J = 2.4 Hz, 1H), 8.07 (dd, J = 8.9, 2.4 Hz, 1H), 7.56 (d, J = 8.2 Hz, 2H), 7.46 (d, J = 8.2 Hz, 2H), 6.80 (d, J = 8.9 Hz, 1H), 2.98 (hep, J = 6.9 Hz, 1H), 1.24 (d, J = 6.9 Hz, 6H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 151.3, 147.5, 145.0, 135.5, 129.0, 128.7, 126.3, 124.6, 124.3, 122.9, 33.20, 23.54. HPLC: purity 97%. MS (IE) (m/z): 307 [M], 309 [M + 2]. Anal. ($C_{15}H_{14}ClNO_2S$) C, H, N, S.

*4-Chloro-2-nitrophenyl 4-isopropylphenyl Sulfide (**27**).* Reagents: 4-isopropyl isopropylthiophenol (0.7 mmol, 1 equiv, 100.0 mg), 2,5-dichloronitrobenzene (0.7 mmol, 1 equiv, 125.9 mg), and sodium acetate (1.0 mmol, 1.5 equiv, 95.5 mg). Solvent: ethanol. Microwave irradiation (85 °C, 1h 30 min). Purification: ethyl acetate:hexane (1:60). Yield: yellow solid (117.1 mg, 60%); mp 108−109 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.29 (d, J = 2.4 Hz, 1H), 7.68 (dd, J = 8.8, 2.4 Hz, 1H), 7.53 (d, J = 8.2 Hz, 2H), 7.43 (d, J = 8.2 Hz, 2H), 6.84 (d, J = 8.8 Hz, 1H), 2.97 (hep, J = 6.9 Hz, 1H), 1.23 (d, J = 6.9 Hz, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 151.0, 145.0, 136.9, 135.4, 134.1, 129.9, 128.5, 125.2, 33.2, 23.5. HPLC: purity 97%. MS (IE) (m/z): 307 [M], 309 [M + 2]. Anal. ($C_{15}H_{14}ClNO_2S$) C, H, N, S.

*4-(4-Chloro-2-nitrophenylthio)aniline (**28**).* Reagents: 4-aminothiophenol (1.2 mmol, 1 equiv, 150.0 mg), 2,5-dichloronitrobenzene (1.2 mmol, 1 equiv, 230.1 mg), and cesium carbonate (1.8 mmol, 1.5 equiv, 585.5 mg). Solvent: acetonitrile. Microwave irradiation (130 °C, 8 min). Purification: ethyl acetate:hexane (1:4). Yield: yellow solid (241.5 mg, 58%); mp 130−131 °C (lit.[36] 127−129 °C). ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.24 (d, J = 2.4 Hz, 1H), 7.65 (dd, J = 8.8, 2.4 Hz, 1H), 7.20 (d, J = 8.6 Hz, 2H), 6.83 (d, J = 8.8 Hz, 1H), 6.67 (d, J = 8.6 Hz, 2H), 5.74 (s, 2H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 151.6, 144.2, 139.9, 137.2, 134.0, 129.1, 129.0, 125.1, 115.2, 111.7. HPLC: purity >99%. MS (ESI+) (m/z): 281 [M + 1], 283 [M + 3]. Anal. ($C_{12}H_9ClN_2O_2S$) C, H, N, S.

*4-Chloro-2-nitrophenyl 4-nitrophenyl Sulfide (**29**).* Reagents: 4-nitrothiophenol (1.0 mmol, 1 equiv, 150.0 mg), 2,5-dichloronitrobenzene (1.0 mmol, 1 equiv, 185.6 mg), and cesium carbonate (1.5 mmol, 1.5 equiv, 472.6 mg). Solvent: acetonitrile. Microwave irradiation (130 °C, 8 min). Purification: ethyl acetate:hexane (1:9). Yield: yellow solid (147.6 mg, 49%); mp 134−135 °C (lit.[37] 135−137 °C). ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.35 (d, J = 2.4 Hz, 1H), 8.29 (d, J = 8.8 Hz, 2H), 7.77 (d, J = 8.8 Hz, 2H), 7.74 (d, J = 8.7, 2.4 Hz, 1H) 7.25 (d, J = 8.7 Hz, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 148.2, 147.9, 141.1, 134.6, 134.0, 133.2, 132.5, 131.7, 125.7, 125.2. HPLC: purity >99%. MS (IE) (m/z): 309 [M], 311 [M + 2]. Anal. ($C_{12}H_7ClN_2O_4S$) C, H, N, S.

*2-Chloro-4-nitrophenyl 4-Nitrophenyl Sulfide (**30**).* Reagents: 4-nitrothiophenol (1.0 mmol, 1 equiv, 150.0 mg), 3-chloro-4-fluoronitrobenzene (1.0 mmol, 1 equiv, 169.7 mg), and cesium carbonate (1.5 mmol, 1.5 equiv, 472.6 mg). Solvent: acetonitrile. Microwave irradiation (130 °C, 8 min). Purification: ethyl acetate:hexane (1:9). Yield: yellow solid (234.0 mg, 78%); mp 146−147 °C (lit.[38] 144 °C). ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.44 (d, J = 2.4 Hz, 1H), 8.29 (d, J = 9.0 Hz, 2H), 8.13 (dd, J = 8.8, 2.4 Hz, 1H), 7.73 (d, J = 9.0 Hz, 2H), 7.36 (d, J = 8.8 Hz, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 147.5, 146.8, 142.2, 139.4, 133.1, 132.9, 131.2, 125.0, 124.8, 123.1. HPLC: purity >99%. MS (IE) (m/z): 309 [M], 311 [M + 2]. Anal. ($C_{12}H_7ClN_2O_4S$) C, H, N, S.

*4-Chloro-2-nitrophenyl Phenyl Sulfide (**31**).* Reagents: thiophenol (1.8 mmol, 1 equiv, 200.0 mg), 2,5-dichloronitrobenzene (1.8 mmol, 1 equiv, 348.5 mg), and cesium carbonate (2.7 mmol, 1.5 equiv, 887.2 mg). Solvent: acetonitrile. Microwave irradiation (130 °C, 8 min). Purification: ethyl acetate:hexane (5:95). Yield: yellow solid (319.1 mg, 66%); mp 81−82 °C (lit.[39] 83−84 °C). ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.27 (d, J = 2.4 Hz, 1H), 7.64 (dd, J = 8.8, 2.4 Hz, 1H), 7.62−7.57 (m, 2H), 7.57−7.51 (m, 3H), 6.82 (d, J = 8.8 Hz, 2H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 145.6, 136.8, 135.7, 134.5, 130.9, 130.8, 130.5, 130.2, 130.1, 125.6. HPLC: purity >99%. MS (IE) (m/z): 265 [M], 267 [M + 2]. Anal. ($C_{12}H_8ClNO_2S$) C, H, N, S.

*2-Chloro-4-nitrophenyl phenyl Sulfide (**32**).* Reagents: thiophenol (1.4 mmol, 1 equiv, 150.0 mg), 3-chloro-4-fluoronitrobenzene (1.4 mmol, 1 equiv, 238.7 mg), and cesium carbonate (2.0 mmol, 1.5 equiv, 665.4 mg). Solvent: acetonitrile. Microwave irradiation (130 °C, 8 min). Yield: gray solid (163.0 mg, 45%); mp 109−110 °C (lit.[40] 117−118.5 °C). ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.35 (d, J = 2.4 Hz, 1H), 8.08 (dd, J = 8.9, 2.4 Hz, 1H), 7.71−7.56 (m, 5H), 6.84 (d, J = 8.9 Hz, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 147.4, 145.5, 135.8, 131.1, 129.7, 128.3, 127.1, 124.7, 123.2. HPLC: purity >99%. MS (IE) (m/z): 265 [M], 267 [M + 2]. Anal. ($C_{12}H_8ClNO_2S$) C, H, N, S.

*4-(4-Chloro-2-nitrophenoxy)aniline (**33**).* Reagents: 4-aminophenol (1.6 mmol, 1.5 equiv, 170.5 mg), 2,5-dichloronitrobenzene (1.0 mmol, 1 equiv, 200.0 mg), and potassium carbonate (2.1 mmol, 2 equiv, 287.5 mg). Solvent: *N*,*N*-dimethylformamide. Microwave irradiation (150 °C, 10 min). Purification: ethyl acetate:hexane (1:6). Yield: yellow solid (177.0 mg, 40%); mp 91−92 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.11 (d, J = 2.7 Hz, 1H), 7.64 (dd, J = 9.1, 2.7 Hz, 1H), 6.90 (d, J = 9.1 Hz, 1H), 6.83 (d, J = 8.8 Hz, 2H), 6.59 (d, J = 8.8 Hz, 2H), 5.15 (s, 2H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 150.5, 146.6, 143.9, 140.1, 134.2, 125.3, 124.8, 120.9, 119.5, 114.8.

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HPLC: purity >99%. MS (ESI+) (m/z): 265 [M + 1], 267 [M + 3]. Anal. ($C_{12}H_9ClN_2O_3$) C, H, N.

*4-(2-Chloro-4-nitrophenoxy)aniline (**34**).* Reagents: 4-aminophenol (1.7 mmol, 1.5 equiv, 186.5 mg), 3-chloro-4-fluoronitrobenzene (1.1 mmol, 1 equiv, 200.0 mg), and potassium carbonate (2.3 mmol, 2 equiv, 314.6 mg). Solvent: *N*,*N*-dimethylformamide. Microwave irradiation (150 °C, 10 min). Purification: ethyl acetate:hexane (1:4). Yield: yellow solid (163.3 mg, 54%); mp 92−93 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.38 (d, J = 2.3 Hz, 1H), 8.13 (dd, J = 8.8, 2.3 Hz, 1H), 6.87 (d, J = 8.8 Hz, 1H), 6.83 (d, J = 8.8 Hz, 1H), 6.63 (d, J = 8.8 Hz, 1H), 5.20 (s, 2H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 160.3, 147.4, 143.7, 141.8, 126.3, 124.9, 122.3, 121.6, 115.9, 115.3. HPLC: purity >99%. MS (ESI+) (m/z): 265 [M + 1], 267 [M + 3]. Anal. ($C_{12}H_9ClN_2O_3$) C, H, N.

*N-[4-(2-Chloro-4-nitrophenoxy)phenyl]acetamide (**35**).* Reagents: 4′-hydroxyacetophenone (1.3 mmol, 1.5 equiv, 194.2 mg), 3-chloro-4-fluoronitrobenzene (0.9 mmol, 1 equiv, 150.0 mg), and potassium carbonate (1.7 mmol, 2 equiv, 235.7 mg). Solvent: *N*,*N*-dimethylformamide. Microwave irradiation (150 °C, 10 min). Purification: ethyl acetate:hexane (1:3). Yield: yellow solid (202.4 mg, 78%); mp 158−159 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.08 (s, 1H), 8.44 (d, J = 2.7 Hz, 1H), 8.15 (dd, J = 9.2, 2.7 Hz, 1H), 7.68 (d, J = 9.0 Hz, 1H), 7.15 (d, J = 9.0 Hz, 1H), 6.95 (d, J = 9.2 Hz, 1H), 2.05 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 168.2, 158.7, 148.9, 142.1, 137.1, 130.0, 126.0, 124.5, 120.7, 120.6, 116.8, 23.9. HPLC: purity >99%. MS (ESI+) (m/z): 307 [M + 1], 309 [M + 3]. Anal. ($C_{14}H_{11}ClN_2O_4$) C, H, N.

*N-[4-(4-Chloro-2-nitrophenoxy)phenyl]acetamide (**36**).* Reagents: 4′-hydroxyacetophenone (1.2 mmol, 1.5 equiv, 177.2 mg), 2,5-dichloronitrobenzene (0.8 mmol, 1 equiv, 150.0 mg), and potassium carbonate (1.6 mmol, 2 equiv, 215.6 mg). Solvent: *N*,*N*-dimethylformamide. Microwave irradiation (150 °C, 10 min). Yield: white solid (145.9 mg, 72%); mp 156−157 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.04 (s, 1H), 8.18 (d, J = 2.6 Hz, 1H), 7.70 (dd, J = 9.1, 2.7 Hz, 1H), 7.63 (d, J = 9.0 Hz, 1H), 7.07 (d, J = 9.1 Hz, 1H), 7.06 (d, J = 9.0 Hz, 1H), 2.03 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 168.2, 149.96, 148.9, 140.9, 136.4, 134.5, 126.7, 125.1, 121.3, 120.7, 119.6, 23.9. HPLC: purity >99%. MS (ESI+) (m/z): 307 [M + 1], 309 [M + 3]. Anal. ($C_{14}H_{11}ClN_2O_4$) C, H, N.

*2-Chloro-4-nitrophenyl 2-Thienyl Sulfide (**37**).* Reagents: 2-thiophenethiol (0.6 mmol, 1 equiv, 75.0 mg), 3-chloro-4-fluoronitrobenzene (0.6 mmol, 1 equiv, 113.5 mg), and potassium carbonate (1.9 mmol, 3 equiv, 267.8 mg). Solvent: tetrahydrofuran. Microwave irradiation (40 °C, 30 min). Purification: ethyl acetate:hexane (1:40). Yield: yellow solid (139.8 mg, 80%); mp 79−80 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.34 (d, J = 2.4 Hz, 1H), 8.13 (dd, J = 8.9, 2.4 Hz, 1H), 8.07 (dd, J = 5.4, 1.3 Hz, 1H), 7.61 (dd, J = 3.6, 1.3 Hz, 1H), 7.34 (dd, J = 5.4, 3.6 Hz, 1H), 6.86 (d, J = 8.9 Hz, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 147.4, 145.8, 139.8, 135.9, 129.9, 129.0, 126.4, 124.7, 124.4, 123.5. HPLC: purity >99%. MS (IE) (m/z): 271 [M], 273 [M + 2]. Anal. ($C_{10}H_6ClN_2O_2S_2$) C, H, N, S.

*4-Chloro-2-nitrophenyl 2-Thienyl Sulfide (**38**).* Reagents: 2-thiophenethiol (0.9 mmol, 1 equiv, 125.0 mg), 2,5-dichloronitrobenzene (1.1 mmol, 1 equiv, 206.8 mg), and potassium carbonate (3.2 mmol, 3 equiv, 446.5 mg). Solvent: tetrahydrofuran. Microwave irradiation (40 °C, 30 min). Purification: ethyl acetate:hexane (1:40). Yield: yellow solid (269.0 mg, 92%); mp 124−125 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.32 (d, J = 2.4 Hz, 1H), 8.04 (dd, J = 5.3, 1.3 Hz, 1H), 7.76 (dd, J = 8.8, 2.4 Hz, 1H), 7.59 (dd, J = 3.5, 1.3 Hz, 1H), 7.33 (dd, J = 5.3, 3.5 Hz, 1H), 6.91 (d, J = 8.8 Hz, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 144.9, 139.7, 137.6, 135.6, 134.9, 130.8, 129.8, 129.1, 126.8, 125.7. HPLC: purity 98%. MS (IE) (m/z): 271 [M], 273 [M + 2]. Anal. ($C_{10}H_6ClN_2O_2S_2$) C, H, N, S.

*2-[(5-Chloro-2-nitrophenyl)thio]-pyridine (**39**).* Reagents: 2-mercaptopyridine (0.9 mmol, 1 equiv, 100.0 mg), 2,4-dichloronitrobenzene (0.9 mmol, 1 equiv, 172.6 mg), and potassium carbonate (0.9 mmol, 1 equiv, 124.1 mg). Solvent: *N*,*N*-dimethylformamide. Microwave irradiation (150 °C, 10 min). Purification: ethyl acetate:hexane (3:7). Yield: yellow solid (37.0 mg, 15%); mp 95−96 °C. ^{1}H NMR (100 MHz, DMSO-d_6): δ 8.59 (dd, J = 4.8, 1.9 Hz, 1H), 8.23 (d, J = 8.8 Hz, 1H), 7.89 (td, J = 7.7, 1.9 Hz, 1H), 7.64 (dd, J = 7.7, 1.1 Hz, 1H), 7.63 (dd, J = 8.8, 2.3 Hz, 1H), 7.43 (ddd, J = 7.7, 4.8, 1.1 Hz, 1H), 7.38 (d, J = 2.3 Hz, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 154.3, 151.2, 146.9, 138.9, 138.8, 133.8, 131.6, 128.5, 127.7, 127.4, 123.9. HPLC: purity 97%. MS (ESI+) (m/z): 267 [M + 1], 269 [M + 3]. Anal. ($C_{11}H_7ClN_2O_2S$) C, H, N, S.

*2-[(4-Chloro-2-nitrophenyl)thio]-pyridine (**40**).* Reagents: 2-mercaptopyridine (1.3 mmol, 1 equiv, 150.0 mg), 2,5-dichloronitrobenzene (1.3 mmol, 1 equiv, 259.1 mg), and potassium carbonate (1.3 mmol, 1 equiv, 186.2 mg). Solvent: *N*,*N*-dimethylformamide. Microwave irradiation (150 °C, 10 min). Yield: yellow solid (332.4 mg, 92%); mp 79−80 °C (lit.[35] 82 °C). ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.54−8.46 (m, 1H), 8.28 (d, J = 2.3 Hz, 1H), 7.83 (td, J = 7.7, 1.9 Hz, 1H), 7.75 (dd, J = 8.6, 2.3 Hz, 1H), 7.52 (dd, J = 7.9, 1.9 Hz, 1H), 7.46 (d, J = 8.6 Hz, 1H), 7.35 (ddd, J = 7.5, 4.8, 1.9 Hz, 1H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 155.1, 150.9, 149.8, 138.6, 135.3, 133.9, 133.1, 128.9, 126.3, 125.4, 123.4. HPLC: purity 97%. MS (ESI+) (m/z): 267 [M + 1], 269 [M + 3]. Anal. ($C_{11}H_7ClN_2O_2S$) C, H, N, S.

*2-[(2-Chloro-4-nitrophenyl)thio]-pyridine (**41**).* Reagents: 2-mercaptopyridine (1.3 mmol, 1 equiv, 150.0 mg), 3-chloro-4-fluoronitrobenzene (1.3 mmol, 1 equiv, 236.9 mg), and potassium carbonate (1.3 mmol, 1 equiv, 186.2 mg). Solvent: *N*,*N*-dimethylformamide. Microwave irradiation (150 °C, 10 min). Yield: brown solid (275.7 mg, 76%); mp 104−105 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.54 (ddd, J = 4.8, 1.9, 0.9 Hz, 1H), 8.40 (d, J = 2.5 Hz, 1H), 8.14 (dd, J = 8.7, 2.5 Hz, 1H), 7.85 (td, J = 7.7, 1.9 Hz, 1H), 7.60 (d, J = 8.7 Hz, 1H), 7.52 (dt, J = 7.7, 1.0 Hz, 1H), 7.38 (ddd, J = 7.7, 4.8, 1.0 Hz, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 153.8, 150.7, 146.9, 141.2, 138.4, 134.2, 132.9, 125.9, 124.6, 123.0, 122.6. HPLC: purity 97%. MS (ESI+) (m/z): 267 [M + 1], 269 [M + 3]. Anal. ($C_{11}H_7ClN_2O_2S$) C, H, N, S.

*4-[(2-Chloro-4-nitrophenyl)thio]-pyridine (**42**).* Reagents: 4-mercaptopyridine (0.9 mmol, 1 equiv, 100.0 mg), 3-chloro-4-fluoronitrobenzene (0.9 mmol, 1 equiv, 157.9 mg), and potassium carbonate (0.9 mmol, 1 equiv, 124.1 mg). Solvent: *N*,*N*-dimethylformamide. Microwave irradiation (150 °C, 10 min). Purification: ethyl acetate:hexane (3:7). Yield: pale-yellow solid (62.0 mg, 52%); mp 133−134 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.58 (d, J = 6.2 Hz, 2H), 8.45 (d, J = 2.4 Hz, 1H), 8.17 (dd, J = 8.7, 2.4 Hz, 1H), 7.53 (d, J = 8.7 Hz, 1H), 7.39 (d, J = 6.2 Hz, 2H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 150.6, 147.2, 142.3, 140.3, 134.2, 132.6, 125.0, 124.9, 123.1. HPLC: purity 97%. MS (ESI+) (m/z): 267 [M + 1], 269 [M + 3]. Anal. ($C_{11}H_7ClN_2O_2S$) C, H, N, S.

*4-[(5-Chloro-2-nitrophenyl)thio]-pyridine (**43**).* Reagents: 4-mercaptopyridine (0.9 mmol, 1 equiv, 100.0 mg), 2,4-dichloronitrobenzene (0.9 mmol, 1 equiv, 172.6 mg), and potassium carbonate (0.9 mmol, 1 equiv, 124.1 mg). Solvent: *N*,*N*-dimethylformamide. Microwave irradiation (150 °C, 10 min). Purification: ethyl acetate:hexane (3:7). Yield: yellow solid (43.0 mg, 45%); mp 149−150 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.64 (d, J = 6.0 Hz, 1H), 8.26 (d, J = 8.9 Hz, 1H), 7.64 (dd, J = 8.9, 2.1 Hz, 1H), 7.52 (d, J = 6.0 Hz, 1H), 7.23 (d, J = 2.1 Hz, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 150.9, 146.3, 142.5, 139.2, 133.8, 130.4, 128.3, 127.7, 126.6. HPLC: purity 97%. MS (ESI+) (m/z): 267 [M + 1], 269 [M + 3]. Anal. ($C_{11}H_7ClN_2O_2S$) C, H, N, S.

*4-[(4-Chloro-2-nitrophenyl)thio]-pyridine (**44**).* Reagents: 4-mercaptopyridine (0.9 mmol, 1 equiv, 100.0 mg), 2,5-dichloronitrobenzene (0.9 mmol, 1 equiv, 172.6 mg), and potassium carbonate (0.9 mmol, 1 equiv, 124.1 mg). Solvent: *N*,*N*-dimethylformamide. Microwave irradiation (150 °C, 10 min). Purification: ethyl acetate:hexane (3:7). Yield: yellow solid (213.0 mg, 88%); mp 106−107 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.58 (d, J = 6.1 Hz, 2H), 8.33 (d, J = 1.9 Hz, 1H), 7.76 (dd, J = 8.7, 1.9 Hz, 1H), 7.43 (d, J = 6.1 Hz, 2H), 7.37 (d, J = 8.7 Hz, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 150.5, 148.9, 143.3, 134.1, 133.9, 132.8, 128.9, 125.6, 125.2. HPLC: purity 97%. MS (ESI+) (m/z): 267 [M + 1], 269 [M + 3]. Anal. ($C_{11}H_7ClN_2O_2S$) C, H, N, S.

*4-(4-Acethyl-2-nitrophenylthio)pyridine (**45**).* Reagents: 4-mercaptopyridine (1.3 mmol, 1 equiv, 150.0 mg), 4-bromo-3-nitroacetophenone (1.3 mmol, 1 equiv, 329.3 mg), and potassium carbonate (1.3 mmol, 1 equiv, 186.2 mg). Solvent: *N*,*N*-dimethylformamide.

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Microwave irradiation (150 °C, 10 min). Purification: methanol:dichloromethane (1:20). Yield: orange solid (146.9 mg, 40%); mp 134−135 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.68 (d, J = 6.0 Hz, 2H), 8.65 (d, J = 2.0 Hz, 1H), 8.12 (dd, J = 8.5, 2.0 Hz, 1H), 7.58 (d, J = 6.0 Hz, 2H), 7.30 (d, J = 8.5 Hz, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 195.7, 162.3, 151.0, 146.5, 141.5, 138.1, 135.2, 133.0, 130.7, 127.6, 125.2, 30.8. HPLC: purity 97%. MS (ESI+) (m/z): 275 [M + 1]. Anal. ($C_{13}H_{10}N_2O_3S$) C, H, N, S.

*4-(Phenylthio)pyridine (**55**).* Reagents: thiophenol (1.8 mmol, 1 equiv, 200.0 mg), 4-bromopyridine hydrochloride (1.8 mmol, 1 equiv, 353.0 mg), and cesium carbonate (3.6 mmol, 2 equiv, 1.18 g). Solvent: acetonitrile. Microwave irradiation (130 °C, 35 min). Purification: ethyl acetate:hexane (1:2). Yield: pale-yellow oil (209.8 mg, 63%). ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.35 (d, J = 6.2 Hz, 2H), 7.68−7.41 (m, 5H), 7.00 (d, J = 6.2 Hz, 2H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 149.6, 149.0, 134.9, 130.3, 129.9, 128.6, 120.5. HPLC: purity 97%. MS (ESI+) (m/z): 188 [M + 1]. Anal. ($C_{11}H_9NS$) C, H, N, S.

*4-(4-Methoxyphenylthio)pyridine (**56**).* Reagents: 4-methoxythiophenol (1.4 mmol, 1 equiv, 200.0 mg), 4-bromopyridine hydrochloride (1.4 mmol, 1 equiv, 225.5 mg), and cesium carbonate (2.1 mmol, 2 equiv, 697.2 mg). Solvent: acetonitrile. Microwave irradiation (130 °C, 35 min). Purification: ethyl acetate:hexane (1:9). Yield: pale-yellow solid (160.4 mg, 53%), mp 96−97 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.32 (d, J = 6.4 Hz, 2H), 7.52 (d, J = 8.6 Hz, 2H), 7.09 (d, J = 8.6 Hz, 2H), 6.92 (d, J = 6.4 Hz, 2H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 166.41, 156.04, 155.07, 155.03, 142.90, 142.86, 125.39, 123.94, 121.53, 121.49, 61.05. HPLC: purity 97%. MS (ESI+) (m/z): 218 [M + 1]. Anal. ($C_{12}H_{11}NOS$) C, H, N, S.

*4-(3-Aminophenylthio)pyridine (**57**).* Reagents: 3-aminothiophenol (1.6 mmol, 1 equiv, 200.0 mg), 4-bromopyridine hydrochloride (1.6 mmol, 1 equiv, 310.8 mg), and cesium carbonate (3.2 mmol, 2 equiv, 1040.0 mg). Solvent: acetonitrile. Microwave irradiation (130 °C, 35 min). Purification: methanol:dichloromethane (1:9). Yield: yellow solid (100.3 mg, 33%); mp 89−90 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.34 (d, J = 6.3 Hz, 2H), 7.14 (t, J = 7.8 Hz, 1H), 7.02 (d, J = 6.3 Hz, 2H), 6.74 (t, J = 2.0 Hz, 1H), 6.69 (dd, J = 8.0, 1.5 Hz, 1H), 6.66 (dd, J = 7.3, 1.5 Hz, 1H), 2.78 (s, 2H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 150.3, 149.8, 149.3, 130.5, 128.3, 121.4, 120.4, 119.2, 115.3. HPLC: purity 97%. MS (ESI+) (m/z): 203 [M + 1]. Anal. ($C_{11}H_{10}N_2S$) C, H, N, S.

*3-(4-Methoxyphenylthio)pyridine (**58**).* Reagents: 4-methoxythiophenol (1.4 mmol, 1 equiv, 200.0 mg), 3-bromopyridine (1.4 mmol, 1 equiv, 225.5 mg), and cesium carbonate (2.1 mmol, 1.5 equiv, 697.2 mg). Solvent: acetonitrile. Microwave irradiation (130 °C, 35 min). Purification: ethyl acetate:hexane (1:2). Yield: pale-yellow oil (180.5 mg, 58%). ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.35 (d, J = 2.4 Hz, 1H), 8.29 (dd, J = 4.8, 1.5 Hz, 1H), 7.36 (d, J = 8.8 Hz, 2H), 7.35 (m, 1H), 7.07 (dd, J = 8.0, 4.7 Hz, 1H), 6.84 (d, J = 8.8 Hz, 2H), 3.76 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 160.7, 149.3, 147.2, 136.4, 136.1, 135.8, 124.1, 123.0, 115.7, 55.8. HPLC: purity 97%. MS (ESI+) (m/z): 218 [M + 1]. Anal. ($C_{12}H_{11}NOS$) C, H, N, S.

General Synthetic Procedure for Compounds 50−54. A mixture of aryl thiol, corresponding 2-halidearylpyridine, and water was heated under microwave irradiation (100 °C, 30 min). After cooling down, ethyl acetate was added (100 mL) and the crude was washed with a saturated NaCl solution (100 mL). The organic phase was dried over magnesium sulfate and chromatographed on silica gel column using as eluents mixtures of solvents in the portions indicated.

*5-Methyl-3-nitro-2-(4-pyridinylthio)pyridine (**50**).* Reagents: 4-mercaptopyridine (1.4 mmol, 1 equiv, 150.0 mg) and 2-chloro-5-methyl-3-nitropyridine (1.4 mmol, 1 equiv, 234.9 mg). Purification: ethyl acetate:hexane (1:4). Yield: orange solid (255.2 mg, 76%); mp 128−129 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.61 (d, J = 6.1 Hz, 2H), 8.52 (m, 2H), 7.54 (d, J = 6.1 Hz, 2H), 2.36 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 154.4, 150.2, 150.0, 141.8, 140.5, 134.2, 132.0, 128.8, 16.7. HPLC: purity 97%. MS (ESI+) (m/z): 248 [M + 1]. Anal. ($C_{11}H_9N_3O_2S$) C, H, N, S.

*2-(4-Pyridinylthio)pyrimidine (**51**).* Reagents: 4-mercaptopyridine (0.9 mmol, 1 equiv, 100.0 mg) and 2-chloropyrimidine (0.9 mmol, 1 equiv, 103.9 mg). Purification: methanol:dichloromethane (1:20). Yield: orange solid (50.2 mg, 30%); mp 82−83 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.67 (d, J = 4.9 Hz, 2H), 8.61 (d, J = 6.1 Hz, 2H), 7.65 (d, J = 6.1 Hz, 2H), 7.34 (t, J = 4.9 Hz, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 168.8, 158.4, 150.0, 140.2, 127.5, 118.8. HPLC: purity 97%. MS (ESI+) (m/z): 190 [M + 1]. Anal. ($C_9H_7N_3S$) C, H, N, S.

*2-[(1,1′-Biphenyl)-4-ylthio]-5-methyl-3-nitropyridine (**52**).* Reagents: biphenyl-4-thiol (0.8 mmol, 1 equiv, 150.0 mg) and 2-chloro-5-methyl-3-nitropyridine (0.8 mmol, 1 equiv, 138.9 mg). Purification: dichloromethane. Yield: yellow solid (89.3 mg, 55%); mp 133−134 °C. ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.49 (s, 1H), 8.48 (s, 1H), 7.74 (d, J = 8.5 Hz, 2H), 7.72 (dd, J = 7.4, 1.2 Hz, 2H), 7.60 (d, J = 8.5 Hz, 2H), 7.49 (t, J = 7.4 Hz, 2H), 7.40 (t, J = 7.4 Hz, 1H), 2.33 (s, 3H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 154.9, 153.2, 141.6, 141.5, 139.6 136.6, 134.5, 131.5, 129.5, 128.9, 128.4, 128.0, 127.3, 17.1. HPLC: purity 97%. MS (ESI+) (m/z): 323 [M + 1]. Anal. ($C_{18}H_{14}N_2O_2S$) C, H, N, S.

*2-(1,1′-Biphenyl)-4-ylthiopyridine (**53**).* Reagents: biphenyl-4-thiol (0.8 mmol, 1 equiv, 150.0 mg) and 2-bromopyridine (0.8 mmol, 1 equiv, 127.2 mg). Purification: ethyl acetate:hexane (1:10). Yield: pale-yellow solid (147.2 mg, 70%); mp 41−42 °C. ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.40 (ddd, J = 4.8, 1.7, 0.8 Hz, 1H), 7.77 (d, J = 8.1 Hz, 2H), 7.71 (dd, J = 7.3, 1.8 Hz, 2H), 7.67 (m, 1H), 7.64 (d, J = 8.1 Hz, 2H), 7.48 (t, J = 7.8 Hz, 2H), 7.39 (t, J = 7.8 Hz, 1H), 7.15 (ddd, J = 7.4, 4.8, 0.8 Hz, 1H), 8.40 (dd, J = 7.4, 0.8 Hz, 1H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 160.1, 150.1, 141.3, 139.5, 137.9, 135.5, 129.8, 129.5, 128.4, 128.3, 127.2, 121.7, 121.1. HPLC: purity 97%. MS (ESI +) (m/z): 264 [M + 1]. Anal. ($C_{17}H_{13}NS$) C, H, N, S.

*5-Methyl-3-nitro-2-(3-aminophenylthio)pyridine (**54**).* Reagents: 3-aminothiophenol (1.2 mmol, 1 equiv, 150.0 mg) and 2-chloro-5-methyl-3-nitropyridine (1.2 mmol, 1 equiv, 206.8 mg). Purification: methanol:dichloromethane (1:30). Yield: yellow solid (155.0 mg, 50%); mp 157−158 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.31 (d, J = 2.1 Hz, 1H), 8.21 (d, J = 2.1 Hz, 1H), 7.15 (t, J = 7.8 Hz, 1H), 6.86 (dt, J = 7.6, 1.4 Hz, 1H), 6.80 (m, 1H), 6.68 (ddd, J = 8.1, 2.3, 1.4 Hz, 1H), 3.66 (s, 2H), 2.29 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 154.6, 147.5, 133.9, 130.8, 130.3, 130.1, 126.2, 122.4, 116.7, 17.7. HPLC: purity 98%. MS (ESI+) (m/z): 262 [M + 1]. Anal. ($C_{12}H_{11}N_3O_2S$) C, H, N, S.

***N*-[4-(4-Chloro-2-nitrophenylthio)phenyl]acetamide (46).** A mixture of 100.0 mg of 4-(4-chloro-2-nitrophenylthio)aniline (**28**) (0.4 mmol, 1 equiv), 0.025 mL of acethyl chloride (0.4 mmol, 1 equiv), and tetrahydrofuran (1 mL) was heated under microwave irradiation (110 °C, 20 min). After cooling down, the solvent was evaporated, and the crude was dissolved in ethyl acetate (100 mL) and was washed with three aqueous solutions: HCl 0.1 M (100 mL), saturated $NaHCO_3$ (100 mL), and saturated NaCl (100 mL). The organic phase was dried over magnesium sulfate and purified by silica gel column chromatography, using ethyl acetate:hexane (1:5) as eluents. Yield: yellow solid (80.0 mg, 70%); mp 197−198 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.24 (s, 1H), 8.28 (d, J = 2.4 Hz, 1H), 7.75 (d, J = 8.6 Hz, 1H), 7.66 (dd, J = 8.8, 2.4 Hz, 1H), 7.54 (d, J = 8.6 Hz, 1H), 6.80 (d, J = 8.8 Hz, 1H), 2.08 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 169.2, 145.7, 145.0, 141.6, 136.9, 134.5, 130.1, 129.8, 125.6, 122.6, 120.8, 24.5. HPLC: purity >99%. MS (ESI+) (m/z): 323 [M + 1], 325 [M + 3]. Anal. ($C_{14}H_{11}ClN_2O_3S$) C, H, N, S.

***N*-[4-(2-Chloro-4-nitrophenylthio)phenyl]acetamide (47).** A mixture of 100.0 mg of 4-(2-chloro-4-nitrophenylthio)aniline (**8**) (0.4 mmol, 1 equiv), 0.025 mL of acethyl chloride (0.4 mmol, 1 equiv), and tetrahydrofuran (1 mL) were heated under microwave irradiation (110 °C, 20 min). After cooling down, the solvent was evaporated, and the crude was dissolved in ethyl acetate (100 mL) and was washed with three aquous solutions: HCl 0.1 M (100 mL), saturated $NaHCO_3$ (100 mL), and saturated NaCl (100 mL). The organic phase was dried over magnesium sulfate, and the product was obtained as a yellow solid (80.5 mg, 70%); mp 166−167 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.27 (s, NH, 1H), 8.31 (d, J = 2.6 Hz, 1H), 8.05 (dd, J = 8.9, 2.6 Hz, 1H), 7.79 (d, J = 8.7 Hz, 2H), 7.56 (d, J = 8.7 Hz, 2H), 6.75 (d, J = 8.9 Hz, 1H), 2.08 (s, CH_3, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 169.2, 148.4, 145.3, 142.0, 136.9, 129.1, 126.5, 124.6, 123.2,

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120.9, 120.7, 24.5. HPLC: purity >99%. MS (ESI+) (m/z): 323 [M + 1], 325 [M + 3]. Anal. ($C_{14}H_{11}ClN_2O_3S$) C, H, N, S.

2-(4-Aminophenylthio)-5-chloroaniline (48). A mixture of 4-(4-chloro-2-nitrophenylthio)aniline (**28**) (0.4 mmol, 1 equiv, 100.0 mg), tin chloride(II) dihydrate (2.0 mmol, 5.5 equiv, 442.1 mg), and 6 mL of ethanol were heated under microwave irradiation (110 °C, 5 min). After cooling down, the solvent was evaporated, and the crude was dissolved in ethyl acetate (100 mL) and was washed with with saturated $NaHCO_3$ (100 mL) and saturated NaCl (100 mL) solutions. The organic phase was dried over magnesium sulfate and purified by silica gel column chromatography using ethyl acetate:hexane (1:3) as eluents. Yield: dark-blue oil (70.0 mg, 78%). ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.01 (d, J = 8.3 Hz, 1H), 7.00 (d, J = 8.5 Hz, 1H), 6.68 (d, J = 2.3 Hz, 1H), 6.48 (d, J = 8.5 Hz, 1H), 6.47 (d, J = 8.3, 2.3 Hz, 1H), 5.47 (s, 2H), 5.26 (s, 2H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 149.6, 149.1, 135.0, 133.4, 133.3, 117.9, 117.7, 116.3, 115.0, 113.7. HPLC: purity >99%. MS (ESI+) (m/z): 251 [M + 1], 253 [M + 3]. Anal. ($C_{12}H_{11}ClN_2S$) C, H, N, S.

4-(4-Aminophenylthio)-3-chloroaniline (49). A mixture of 4-(2-chloro-4-nitrophenylthio)aniline (**8**) (0.4 mmol, 1 equiv, 100.0 mg), tin chloride(II) dihydrate (2.0 mmol, 5.5 equiv, 442.1 mg), and ethanol (6 mL) were heated under microwave irradiation (110 °C, 5 min). After cooling down, the solvent was evaporated, and the crude was dissolved in ethyl acetate (100 mL) and was washed with saturated $NaHCO_3$ (100 mL) and saturated NaCl (100 mL) solutions. The organic phase was dried over magnesium sulfate and purified by silica gel column chromatography using ethyl acetate:hexane (1:3) as eluents. Yield: dark-yellow oil (102.3 mg, 76%). ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.00 (d, J = 8.5 Hz, 1H), 6.82 (d, J = 8.4 Hz, 1H), 6.63 (d, J = 2.2 Hz, 1H), 6.52 (d, J = 8.5 Hz, 1H), 6.42 (dd, J = 8.4, 2.2 Hz, 1H), 5.45 (s, 2H), 5.32 (s, 2H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 149.7, 149.2, 134.7, 134.0, 133.4, 120.4, 118.5, 115.0, 114.5, 113.9. HPLC: purity >99%. MS (ESI+) (m/z): 251 [M + 1], 253 [M + 3]. Anal. ($C_{12}H_{11}ClN_2S$) C, H, N, S.

Radiometric Phosphodiesterase Inhibition Assay. The methodology used for measuring human recombinant PDE7A1, PDE7B, PDE8A, PDE10A2, and PDE3A activity was based in a scintillation proximity assay (SPA) from PerkinElmer (TRKQ7090). The activity of the phosphodiesterase was measured by coincubating the enzyme with [^{3}H]cAMP, and the hydrolysis of the nucleotide was quantified by radioactivity measurement after binding of [^{3}H]AMP to scintillation binding bead.

Either 0.02 units of PDE7A1 (Calbiochem no. 524751), 0.02 units of PDE3A (Calbiochem no. 524742), 0.02 units of PDE10A (Calbiochem no. 524739), 0.5 units of PDE7B (Abcam no. ab79800), or 0.5 units of PDE8A (Abcam no. ab125540) were incubated in a 96-well flexiplate with 5 nCi of [^{3}H]cAMP and inhibitors in 100 μL of assay buffer (contained in the kit) for 20 min at 30 °C. After the incubation time, 50 μL of a solution of SPA-beads (approximately 1 mg per well) were added to each well, and the plate was shaken for 1 h at room temperature. Finally, beads were settled for 30 min and radioactivity was detected in a Microbeta Trilux reader. IC_{50} values were calculated by nonlinear regression fitting using GraphPad Prism. Data (radioactivity vs log concentration) was fitted to a sigmoidal dose−response equation: Y = Bottom + (Top-Bottom)/(1 + $10^{((\log IC_{50}-X)\times n))}$, where Bottom and Top were the minimum and maximal inhibition for PDE, respectively, IC_{50} was the concentration of compound that inhibited the PDE activity in a 50%, and n was the slope of the concentration−response curve.

The mode of inhibitory action for compound **28** and **42** was determined by varying the concentration of unlabeled cAMP in the reaction cocktail within the range of 5 nM to 2 μM in the presence of a fixed concentration of [^{3}H]-cAMP tracer and inhibitor concentrations. Enzyme activity data were analyzed using Lineweaver−Burk plots using GraphPad Prism.

Fluorescence Polarization Phosphodiesterase Inhibition Assay. PDE4B2, PDE4D3, PDE1A, PDE5, and PDE9 phosphodiesterases showed a little signal/background ratio when they were screened by using the radiometric assay. To increase this signal window, they were screened by using IMAP fluorescence polarization (FP) assay (Molecular Devices R8175).

First, 0.05U of PDE4B2 (Calbiochem no. 524736), 0.05 units of PDE4D3 (Calbiochem no. 524733), 0.15 units of PDE5 (Calbiochem no. 524738), 2 units of PDE1 (Abcam @ab125661), and 0.05 units of PDE9 (Abcam no. ab54113) were incubated in a 96-well black half area plate with 1 nM of either fluorescein adenosine 3′,5′-cyclic phosphate or fluorescein guanosine 3′,5′-cyclic phosphate (PDE5) contained in the kit and inhibitors in 40 μL of assay buffer. Then plates were mixed on a shaker for 10 s and incubated at ambient temperature for 60 min. IMAP binding reagent was added (60 μL of a 1 in 600 dilution in binding buffer of the kit stock solution) to terminate the assay. Plates were allowed to stand at ambient temperature for 1 h. The FP ratio was measured with a Tecan Ultra Evolution reader.

IC_{50} values were calculated by nonlinear regression fitting using GraphPad Prism. Data (fluorescence polarization vs log concentration) was fitted to a sigmoidal dose−response equation: Y = Bottom + (Top − Bottom)/(1 + $10^{((\log IC_{50}-X)\times n))}$, where Bottom and Top were the minimum and maximal inhibition for PDE, respectively, IC_{50} was the concentration of compound that inhibited the PDE activity in a 50%, and n was the slope of the concentration−response curve.

Docking Studies. To carry out the docking studies, the PDE7A enzyme employed was based on the crystal structure reported in the Protein Data Bank (PDB code 1ZKL). IBMX, a well-known inhibitor of PDE, whose crystal structure in complex with the enzyme is reported in the Protein Data Bank (PDB code 1ZKL), was docked as reference compound. Protein was prepared for docking using Protein Prepared Wizard (Schrodinger Inc.). Water molecules coordinated with both magnesium and zinc atoms were conserved for the docking development. Hydrogens were incorporated at neutral pH, and the whole structure was minimized using force field OPLS2005.

Ligands were prepared using Ligprep (Schrodinger Inc.). The ligands were minimized using OPLS2005 force field, and all the possible states at target pH 5−9 were generated. The lowest energy conformer for every molecule were docked in the PDE7A protein (PDB code1ZKL) by using the automatic docking program Glide (Schrodinger Inc.). Glide was used to generate a grid based on the centroid of select residues in the binding pocket (Phe384, Gln413, and Phe416). The box size was set to the value of 20 × 20 × 20 Å^3. This box size encompasses the entire PDE7A binding pocket both in width and depth. Extra precision (XP) mode and flexible docking with ring sampling were selected for the docking setup. After docking, the receptor−ligand complexes were minimized using the OPLS2005 all atom force field.

SH-SY5Y Cell Culture. The human neuroblastoma SH-SY5Y cell line (Sigma-Aldrich) was cultured in F12 medium/EMEM containing 2 mM glutamine, 1% nonessential amino acids, and 15% fetal bovine serum (FBS) under standard culture conditions (37 °C and 5% CO_2). On attaining semiconfluence, cells were pretreated for 1 h with the different compounds at several concentrations (30 μM for BRL50481 and 10 μM for the rest of tested compounds). After that, 6-OHDA (35 μM, Sigma) was added to the cultures and incubated for 16 h. Finally, cultures were processed for cell viability assay. Some cultures were treated for 16 h only with the compounds **28** and **42** for Western blot analysis.

Primary Cell Cultures. Glial cells were prepared from neonatal rat cerebral cortex, as previously described by Luna-Medina et al.[41] Briefly, after removal of the meninges, the cerebral cortex was dissected, dissociated, and incubated with 0.25% trypsin/EDTA at 37 °C for 1 h. After centrifugation, the pellet was washed 3 times with HBSS (Gibco) and the cells were plated on noncoated flasks and maintained in HAMS/DMEM (1:1) medium containing 10% FBS. After 15 days, the flasks were agitated on an orbital shaker for 4 h at 240 rpm at 37 °C, the supernatant was collected, centrifuged, and the cellular pellet containing the microglial cells resuspended in complete medium (HAMS/DMEM (1:1) containing 10% FBS) and seeded on uncoated 96-well plates. Cells were allowed to adhere for 2 h, and the medium was removed to eliminate nonadherent oligodendrocytes. New fresh medium containing 10 ng/mL of GM-CSF was added. The remaining astroglial cells adhered on the flasks were then trypsinized, collected,

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centrifuged, and plated onto 96-well plates with complete medium. The purity of cultures obtained by this procedure was >98% as determined by immunofluorescence with the OX42 (microglial marker) and the GFAP (astroglial marker) antibodies. After 2 days in culture, cells were pretreated for 2 h with BRL50481 (30 μM), and the different compounds at 10 μM were also treated with lipopolysaccharide (LPS; 10 μg/mL) for 24 h. Nitrite production on cultures was then measured.

Cell Viability Assay. Cell viability was measured using the MTT assay from Roche, based on the ability of viable cells to reduce yellow MTT to blue formazan. Cells cultured for 16 h were incubated with MTT (0.5 mg/mL, 4h) and subsequently solubilized in 10% SDS/0.01 M HCl for 1 h in the dark. The extent of reduction of MTT was quantified by absorbance measurement at 595 nm according to the manufacturer's protocol.

Western Blot Analysis. Proteins were isolated from cell cultures following standard methods. Briefly, cells were resuspended in ice-cold cell lysis buffer (Cell Signaling Technology) with protease inhibitor cocktail (Roche) and incubated for 15−30 min on ice. A total amount of 30 μg of protein was loaded on a 12% SDS-PAGE gel, and after electrophoresis, proteins were transferred to nitrocellulose membranes (Protran, Whatman) and blots probed with primary antibodies against p-CREB (rabbit; Cell Signaling) and CREB (rabbit; Cell Signaling). Secondary peroxidase-conjugated donkey antirabbit (Amersham Biosciences, GE Healthcare) antibody was used. Values in the figure are the average of the quantification of at least three independent experiments corresponding to three different samples.

Nitrites Measurement. Accumulation of nitrites in media was assayed by the standard Griess reaction. After stimulation of cells with LPS during 24 h, supernatants were collected and mixed with an equal volume of Griess reagent (Sigma). Samples were then incubated at room temperature for 15 min and absorbance read using a plate reader at 492/540 nm.

CNS Penetration: In Vitro Parallel Artificial Membrane Permeability Assay (PAMPA) Blood−Brain Barrier (BBB). Prediction of the brain penetration was evaluated using a parallel artificial membrane permeability assay (PAMPA).[22] Ten commercial drugs, phosphate buffer saline solution at pH 7.4 (PBS), ethanol, and dodecane were purchased from Sigma, Acros Organics, Merck, Aldrich, and Fluka. The porcine polar brain lipid (PBL) (catalogue no. 141101) was from Avanti Polar Lipids. The donor plate was a 96-well filtrate plate (Multiscreen IP sterile plate PDVF membrane, pore size is 0.45 μM, catalogue no. MAIPS4510), and the acceptor plate was an indented 96-well plate (Multiscreen, catalogue no. MAMCS9610) both from Millipore. Filter PDVF membrane units (diameter 30 mm, pore size 0.45 μm) from Symta were used to filtered the samples. A 96-well plate UV reader (Thermoscientific, Multiskan spectrum) was used for the UV measurements. Test compounds [(3−5 mg of caffeine, enoxacine, hydrocortisone, desipramine, ofloxacine, piroxicam, and testosterone), (12 mg of promazine), and 25 mg of verapamil and atenolol] were dissolved in EtOH (1000 μL). Then 100 μL of this compound stock solution was taken and 1400 μL of EtOH and 3500 μL of PBS pH 7.4 buffer were added to reach 30% of EtOH concentration in the experiment. These solutions were filtered. The acceptor 96-well microplate was filled with 180 μL of PBS/EtOH (70/30). The donor 96-well plate was coated with 4 μL of porcine brain lipid in dodecane (20 mg mL^{-1}), and after 5 min, 180 μL of each compound solution was added. Then 1−2 mg of every compound to have their ability to pass the brain barrier determined were dissolved in 1500 μL of EtOH and 3500 μL of PBS pH 7.4 buffer, filtered, and then added to the donor 96-well plate. Then the donor plate was carefully put on the acceptor plate to form a "sandwich", which was left undisturbed for 2 h and 30 min at 25 °C. During this time, the compounds diffused from the donor plate through the brain lipid membrane into the acceptor plate. After incubation, the donor plate was removed. A UV plate reader determined the concentration of compounds and commercial drugs in the acceptor and the donor wells. Every sample was analyzed at 3−5 wavelengths, in three wells, and in two independent runs. Results are given as the mean [standard deviation (SD)], and the average of the two runs were reported. Ten quality control compounds (previously mentioned) of known BBB permeability were included in each experiment to validate the analysis set.

Emesis Surrogate Model. In vivo experiments were performed using CD1 male mice (25−30 g) purchased in Charles Rivers (France). Mice were hosed in a temperature (21 ± 1 °C) and humidity (60 ± 20%) controlled environment and grouped four per cage. Food and water were available ad libitum. A period of 1 week to habituation to colony facilities was allowed prior to experiments. Procedures were conducted according to standard ethical guidelines (European Communities Council Directive 86/609 EEC) and approved by the local ethical committee (CEEA−PRBB). All in vivo experiments were performed in the colony facilities of the University Pompeu Fabra (Ubiomex−PRBB). To select the doses of BRL50481 (and rolipram and roflumilast) for the in vivo study, a preliminary set of experiments was performed on a surrogate model for emesis potential in mice (data not shown). In the case of BRL50481, the doses assayed were 0.1, 1, 5, and 10 mg/kg (sc) in mice. Any of the doses used reversed the anesthetic effect of the mixture of ketamine/xylacine in mice. Because only one dose of BRL50481 was shown in the manuscript, we selected for that, the dose of 5 mg/kg that is high enough to discard an emetic effect of this compound.

The selected compound **28** shows an inhibitory effect on PDE7 of 81% at a concentration of 10 μM while producing a light effect on PDE4 (4% of inhibition). Considering this residual effect on PDE4 activity, we selected a range of relative high doses of the compound **28** (2.5, 5, and 10 mg/kg) in order to completely discard a possible emetic effect and, in consequence, this undesirable side effect of the compound **28**.

Experiments were performed by following procedures previously reported[19] and adapted to our experimental conditions. Briefly, mice were anesthetized with the mixture solution of xylacine (10 mg/kg) and ketamine (100 mg/kg) administered by intraperitoneal (ip) route. Fifteen minutes later, the mice were injected subcutaneously (sc) with the evaluated compound, sulfide **28**, at three different doses (2.5, 5, and 10 mg/kg) or the vehicle, polyethylene glycol (PEG). All compounds were freshly dissolved in 60% (v/v) PEG (in saline) and administered in a volume of injection of 0.1 mL/10 g body weight. Three different compounds were used as respective controls: (R), rolipram (1 mg/kg, sc); roflumilast (1 mg/kg, sc); and BRL50481 (5 mg/kg, sc). Rolipram and roflumilast were purchased in Sigma Chemical Co (Madrid, Spain). BRL50481 was purchased in Tocris. Rolipram and roflumilast are reference inhibitors of PDE4, exhibiting emetic-related effects, whereas BRL50481 is an inhibitor of PDE7 showing no emetic-related effects. The return of the righting reflex (when the mouse backed and turned itself spontaneously to prone position) was used as an end point to measure the duration of anesthesia. The duration of anesthesia is expressed as the mean ± SEM. Statistically significant differences among groups were analyzed using ANOVA, and a subsequent posthoc analysis was applied when required (Dunnett test). The level of significance in all the studies was $p < 0.05$.

In our experimental conditions, the combined administration of xylacine (10 mg/kg) and ketamine (100 mg/kg) induced loss of righting reflex within 15 min following injection. The overall duration of anesthesia measured was 80 ± 7.1 min ($n = 10$). One-way ANOVA for the duration of the anesthesia calculated for the different compounds evaluated exhibited significant effects ($F(6, 107) = 4.617$; $p < 0.01$; $n = 10−19$ per group).

Three additional PDE7 inhibitors were evaluated in order to support data obtained with the compound **28**: the quinazoline derivative TC3.6, the furan derivative MR1.51, and the iminothiadiazole VP1.15. In vivo experiments for evaluating these compounds were performed in a different set of experiments and conducted as above-described for the compound **28**. The only difference in the methodology was that mice were anesthetized with the mixture solution of xylacine (10 mg/kg) and ketamine (80 mg/kg). In this conditions, the overall duration of anesthesia measured was 120 ± 16.1 min ($n = 10$).

For the in vivo experiments, data were analyzed using One-Way ANOVA and subsequent posthoc analysis (Scheffe's test) when required.

■ ASSOCIATED CONTENT

Supporting Information

Elemental analyses of all synthesized compounds, Linear correlation between experimental and reported permeability of commercial drugs using the PAMPA-BBB assay, and permeability effective (Pe 10^{-6} cm·s^{-1}) in the PAMPA-BBB assay for 10 commercial drugs, used in the experiment validation, and different sulfide-like PDE7 inhibitors with their predictive penetration in the CNS. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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