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Synthesis, biological evaluation and molecular modelling studies on benzothiadiazine derivatives as PDE4 selective inhibitors

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Abstract—A series of 2,1,3- and 1,2,4-benzothiadiazine derivatives (BTDs) were synthesized and evaluated for their inhibitory activity versus enzymatic isoforms PDE3, PDE4 and PDE7. The compounds characterized by the 3,5-di-*tert*-butyl-4-hydroxybenzyl moiety at N1 position of 2,1,3-benzothiadiazine core (**8**, **13**, **18**), were found active and selective at micromolar level versus PDE4 and could be studied as new leads for the treatment of asthma and COPD (Chronic Obstructive Pulmonary Disease). The antioxidant activity evaluation on the same compounds highlighted **13** as the most significative. Molecular modelling studies gave further support to biological results and suggested targeted modifications so as to improve their potency.

1. Introduction

The function of many inflammatory cells is controlled by cyclic nucleotides, such as cyclic AMP and cyclic GMP, both of which are inactivated by phosphodiesterases (PDEs).[1,2] This suggested the possibility that PDE inhibitors may display beneficial anti-inflammatory activity. In fact, the inhibition of PDE4 and PDE7, two of the known enzymatic isoforms, leads to interruption of inflammatory process present in some diseases such as asthma and COPD (Chronic Obstructive Pulmonary Disease), symptomatically characterized by a repeated stridor and paroxysm due to the airway contraction.[3–7]

Rolipram is the prototypic of the PDE4 inhibitors but nausea and emetic effects limit its therapeutic potential. In the last years both Rolipram related and unrelated second-generation PDE4 inhibitors belonging to very different chemical classes were developed. Roflumilast and Cilomilast appear to display favourable anti-asthma and anti-COPD properties and are currently in phase III clinical trials.[8–11]

It is known from the literature that benzothiadiazine derivatives (BTDs) are heterocyclic inhibitors of PDE7 with concurrent inhibitory activity at PDE4 and PDE3.[12,13]

In the present study we synthesized N-3 mono (**1**–**6**) and N-1,3 disubstituted (**7**–**19**) 2,1,3-BTDs and N-2 substituted 1,2,4-BTDs (**20**–**25**).

Some of the N-substituents here considered such as methylphthalimide, nitrophenyl or 2,6-di-*tert*-butylphenol were present in PDE4 inhibitors such as nitraquazone, CDC-801 is a thalidomide analogue, and benzoxazole derivatives recently patented by Euroceltique (Chart 1).[14] Then we evaluated the PDE4 inhibitory activity (pIC_{50}) and isoenzyme selectivity versus PDE3 and PDE7 of the new compounds.

Moreover, it is known that the antioxidant 2,6-di-*tert*-butylphenol moiety characterizes dual 5-lipoxygenase (5-LO)/cyclooxygenase 2 (COX-2) inhibitors with anti-inflammatory activities.[15–18] COX catalysis involves radical intermediates and a radical scavenging moiety interferes with the cyclooxygenase reaction. To

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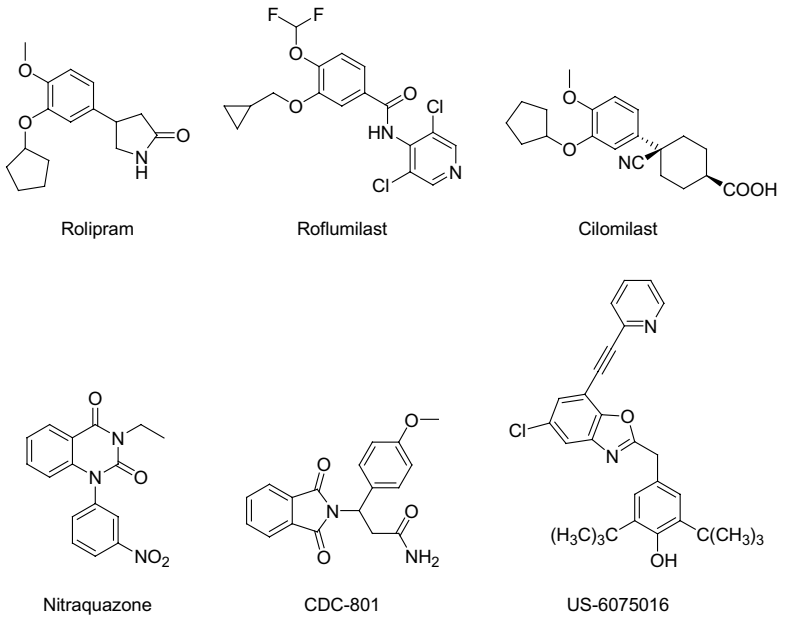


Chart 1.

acquire preliminary information about the antioxidant activity of synthesized compounds, the radical scavenging effect was determined in the presence of DPPH.

Finally docking studies were carried out on the most active compounds as further investigation on the molecular interactions that lead to the PDE4 inhibition.

2. Results

2.1. Chemistry

Scheme 1 outlines the synthesis of 2,1,3-BTDs. The N-3 substituted 2,1,3-BTDs (**1**–**6**) (Table 1) were obtained starting from the suitable methyl 2-aminobenzoate and sulfamoyl chloride.[19,20] The intermediate *N*-sulfamoylanthranilates were cyclized by sodium methoxide. By alkylation with the appropriate alcohol, via Mitsunobu reaction,[21] the N,N-disubstituted derivatives (**7**–**19**) (Table 1) were obtained.

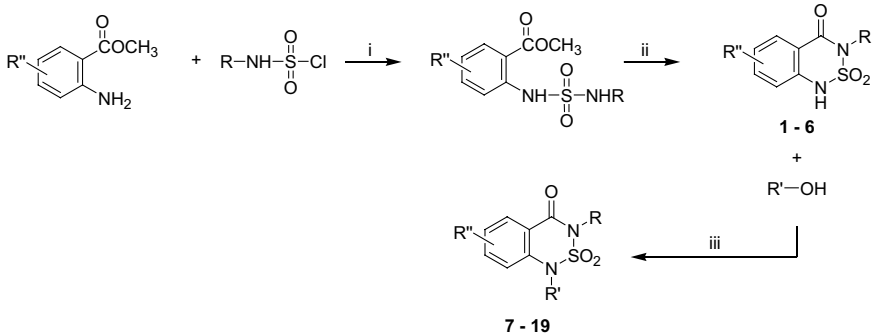
The 1,2,4-BTDs were prepared by condensation of 2-amino-4-chlorobenzensulfonamide with chloroacetaldehyde in aqueous solution[22] and subsequent Mitsunobu alkylation of intermediate **26** (Scheme 2, Table 2). NOESY experiments showed that the alkylation of **26** took place at N-2; thus, proton of NH in position-4 presents NOE effect with aromatic H-5.

The structures of all new compounds were elucidated from their analytical and spectroscopic data reported in Experimental. Unequivocal assignments of ^{1}H NMR chemical shifts were done using bi-dimensional experiments such as COSY, HMBC and HMQC.

2.2. Biology

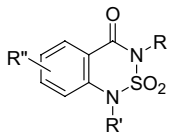
Table 3 shows the in vitro inhibitory activity of BTDs with respect to PDE3 from cytosol of human platelets and to human recombinant enzymes PDE4D3 and PDE7A1 from Baculovirus/Sf21 insect cell system. The 3-isobutyl-1-methylxanthine (IBMX) was taken as non-selective reference compound. All data are expressed as pIC_{50}.

Among the tested compounds, only the 2,1,3-benzothiadiazines with the 3,5-di-*tert*-butyl-4-hydroxybenzyl moi-

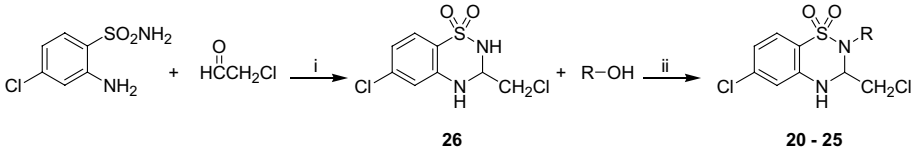


Scheme 1. Reagents and conditions: (i) toluene, Et_3N, 80 °C, 1 h; (ii) Na, CH_3OH, 40 °C, 2 h; (iii) TPP, DIAD, THF, N_2, rt.

Table 1. Compounds **1**–**19**



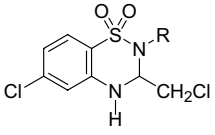
Compd	R	R′	R″
1[30]	Isopropyl	H	H
2	Propyl	H	6,7-Dimethoxy
3[31]	Benzyl	H	H
4	Benzyl	H	7-Cl
5	Benzyl	H	6,7-Dimethoxy
6[32]	2-Phenylethyl	H	H
7	Isopropyl	4-OH-Benzyl	H
8	Isopropyl	3,5-Di-*tert*-butyl-4-OH-benzyl	H
9	Isopropyl	3-Nitro-benzyl	H
10	Isopropyl	Cyclohexyl	H
11[33]	Isopropyl	*N*-Methylphthalimide	H
12	Isopropyl	Ethyl-pyrrolydine-2,5-dione	H
13	Propyl	3,5-Di-*tert*-butyl-4-OH-benzyl	6,7-Dimethoxy
14	Benzyl	4-OH-Benzyl	H
15	Benzyl	3-Nitro-benzyl	H
16[32]	Benzyl	2-Furylmethyl	H
17[32]	Benzyl	*N*-Methylphthalimide	H
18	Benzyl	3,5-Di-*tert*-butyl-4-OH-benzyl	7-Cl
19	2-Phenylethyl	2-Nitro-benzyl	H



26 **20 - 25**

Scheme 2. Reagents and conditions: (i) NH_4Cl, DMF, H_2O, 100 °C, 1 h; (ii) TPP, DIAD, THF, N_2, rt, overnight.

Table 2. Compounds **20**–**25**



Compd	R
20	4-OH-Benzyl
21	4-Pyridylmethyl
22	2-Nitro-benzyl
23	3-Nitro-benzyl
24	*N*-Methylphthalimide
25	*N*-Ethyl-pyrrolydine-2,5-dione

ety at N1 and an isopropyl (**8**), propyl (**13**) and benzyl (**18**) group at N3 showed PDE inhibitory activity (IC_{50} between 1 and 10 μM) with an interesting PDE4 selectivity. The removal of 3,5-di-*tert*-butyl group (**7**, **14**) leads to a marked decrease of the activity so as the substitution of 3,5-di-*tert*-butyl-4-hydroxybenzyl moiety with other aromatic (**9**, **15**, **19**) or aliphatic groups (**10**, **12**, **16**).

2.3. Molecular modelling studies

In order to further rationalize the biological results, a molecular docking study was carried out on compounds **8**, **13**, **18**, the most active as PDE4 inhibitors among those newly synthesized. The compounds were docked into PDE4D catalytic domain[23] (PDB entry 1MKD) using the FlexX[24] module as implemented in Sybyl.[25] FlexX is a widely used docking algorithm in drug design whose ability in predicting a conformation of the ligand very close to its X-ray structure has been widely described in literature.[26]

According to our FlexX calculations, compounds **8**, **13**, **18** could bind to the PDE4D catalytic site occupying part of the pocket where zardaverine, the co-crystallized ligand, binds. This region, defined by Lee et al.[23] as the inhibitor binding pocket, is delimited mainly by the following hydrophobic residues: Phe469, Leu416, Met370, Met434, Ile433, Phe437 and Met454. Zardaverine fills approximately half of the active site-pocket, while more selective and chemically different PDE4 inhibitors such as roflumilast, according to Lee, are able to better occupy this site. For clarity, in the description of interactions of our compounds with PDE4D catalytic site, we have

Table 3. In vitro PDE3/4/7 inhibitory activity

Compd	PDE3[a] pIC_{50}[c]	PDE4[b] pIC_{50}[c]	PDE7[b] pIC_{50}[c]
IBMX	5.3	5.0	4.0
3	<4.0	<4.0	<4.0
4	<4.0	<4.0	<4.0
5	<4.0	<4.0	<4.0
7	<4.0	<4.0	<4.0
8	<4.0	**5.3**	<4.0
9	<4.0	4.4	<4.0
10	<4.0	<4.0	<4.0
11	<4.0	<4.0	<4.0
12	<4.0	<4.0	<4.0
13	<4.0	**5.4**	4.2
14	<4.0	<4.0	<4.0
15[d]	<5.0	<5.0	<5.0
16[d]	<5.0	<5.0	<5.0
17[d]	<5.0	<5.0	<5.0
18	<4.0	**5.3**	<4.0
19[d]	<5.0	<5.0	<5.0
20	<4.0	4.3	<4.0
21	<4.0	4.3	<4.0
22	<4.0	<4.0	<4.0
23	<4.0	<4.0	<4.0
24	<4.0	<4.0	<4.0
25	<4.0	<4.0	<4.0

[a] Inhibition of PDE3 was investigated in the cytosol of human platelets.
[b] The activity against PDE4 and PDE7 was determined versus human recombinant enzymes PDE4D3 and PDE7A1.
[c] $pIC_{50} = -\log IC_{50}$.
[d] 10^{-4} insoluble.

divided the inhibitor binding site into three main sub-pockets, S1, S2 and S3, as proposed by Lee et al.[23] The S1 sub-pocket is formed by Met434, Met454 and Phe437. The sub-pocket S2 consists of Met370, Ser371, Glu327, Asn306, Asp369 and metal ions. The S3 sub-pocket branches from the middle of the main active site pocket and is characterized by residues Phe437, Glu436, Gln440, Ser305, Cys455 and Ser452.

Compounds **8**, **13**, **18** mainly interact with S2 sub-pocket, in particular with Met370, a key residue for the selectivity towards PDE4D, and with Asn306. Molecular interactions displayed by **8**, **13**, **18** are reported in Table 5. More in details, the main features of the binding mode proposed for compound **8** are two hydrogen bonds, the first between the carbonyl group on position-4 of 2,1,3-BTDs and the NH of His301 side chain, the second between an oxygen of the sulfonic function and the NH of Asn306 backbone. In the case of **13** (Fig. 2), the best solution proposed by FlexX presents

Table 4. Absorbance decrease at 514 nm versus control (ΔA) and (S.A.%) for 1.0×10^{-4} M solutions after 6 h

Compd	ΔA[a]	S.A.%[b]
8	0.2007 ± 0.00527	39.02
13	0.3131 ± 0.00546	60.87
18	0.2213 ± 0.01359	43.02

[a] The means values were obtained from quadruplicate experiments.
[b] S.A.% = $100 \times \Delta A/A_t$ where A_t is absorption at 514 nm of DPPH solution (control) after 6 h.

Table 5. Molecular interactions displayed by compounds **8**, **13** and **18** in the top-ranked docked conformations

Compound	H-bonds
Roflumilast	His301, Met370
Cilomilast	His261, His297, Asp298, His301
8	His301, Asn306
13	Asn306, Gln307, Met370
18	His301, Asn306, Met370

a H-bond between carbonyl group on position-4 of the ligand and the NH of Gln307 backbone, plus two H-bonds between the methoxy groups on positions-6 and -7 and the NH of Asn306 and Met370 backbone, respectively. An alternative binding mode proposed by FlexX for **13** is similar to the one of **8**, via two H-bonds between the carbonyl group on position-4 of the ligand and the NH of His301 side chain and between an oxygen on the sulfonic moiety and the NH of Met370 backbone. According to the biological data available in the SWISS-PROT database[27] and the results of multiple sequence alignments of PDE isozymes performed by us employing CLUSTALW,[28] Met370 is one of few residues in the catalytic site with significant sequence variation in known PDEs. A specific interaction of our compounds via H-bond with this residue, could thus help in explaining their selectivity towards PDE4.

In our model, the benzothiadiazine **18**, which presents a bulkier substituent on N-3 in comparison with **8** and **13**, follows the same binding pattern, displaying three hydrogen bonds with His301, Asn306 and Met370. Interestingly, two of these H-bonds involve the oxygens of the sulfonic group. Surprisingly, even if the inhibitor binding pocket presents several hystidine and phenylalanine residues and the new BTDs possess two or three aromatic rings, no π–π interactions were observed.

In order to point out significant differences in the binding mode among **8**, **13**, **18** and more potent PDE4 inhibitors, Cilomilast (Ariflo) and Roflumilast were docked into the enzyme active site (Table 5). In this way we were able also to compare FlexX results with Lee hypothesis on their binding pose.

In agreement with Lee, Roflumilast and Cilomilast, characterized by bulkier groups replacing the methoxy moiety on the dialkoxy pharmacophore, an essential feature of all PDE4 inhibitors, were found to occupy S1 area of the catalytic site. Besides, having a more elongated structure than zardaverine, they resulted able to fill also the sub-pocket S2, displaying a hydrophobic interaction with Met370. In this sub-region, a good superimposition of roflumilast and compound **13** has however been observed (Fig. 2).

3. Discussion

The biological results show the important role of the 3,5-di-*tert*-butyl-4-hydroxybenzyl moiety in N1 of 2,1,3-benzothiadiazines in eliciting PDE4 inhibitory activity. The molecular docking study into PDE4D cat-

alytic domain indicates that, probably, the bulky di-*tert*-butyl groups, which protrude at the top of S3 sub-pocket, contribute to better stabilize the molecule into the catalytic site of the enzyme. All the other compounds, lacking of this moiety, are unable to interact with the amino acid residues of S3. Furthermore, the 6,7-dimethoxy substitution at the aromatic ring of benzothiadiazine core (**13**) leads to the most potent compound (pIC_{50} 5.4); the structure of part of this molecule mimics the well known di-alkoxy aryl moiety common to a wide number of PDE4 inhibitors like Rolipram, Cilomilast and Roflumilast.

The 2,1,3-BTD derivatives **11** and **17**, with a N1-methylphthalimide substitution, as in some PDE4 inhibitors structurally related to Thalidomide,[14] completely lacked of activity. This could be explained, on the basis of our computational results, taking into account the high hydrophobicity of S3 site where N1 substituent should be allocated. The hydrophilicity of the methylphthalimide moiety could probably determine unfavourable interactions. All novel synthesized compounds are unable to inhibit PDE3 or PDE7.

The 2,1,3-BTDs N3 substituted (**3**–**5**) and the 1,2,4-BTDs N2 substituted (**20**–**25**) are unable to significantly inhibit the PDE4 enzyme. For this reason we can assert that, in the case of 2,1,3-BTD heterocyclic system, the double N,N substitution is necessary for activity, while the synthesis of N,N disubstituted 1,2,4-BTDs must be performed to elucidate the S.A.R. of this class of benzothiadiazine derivatives.

The 2,1,3-BTD derivatives **8**, **13**, **18** are also characterized by antioxidant properties. UV measurement of free radical scavenging activity (S.A.%)[29] showed that these compounds scavenge the DPPH radical. The DPPH absorbance shows a nonlinear exponential decay (Fig. 1).

The S.A.% of each compound was expressed by the ratio of absorbance decrease of DPPH solution in presence of compound (ΔA) versus control (absorbance of DPPH solution in the absence of compound) (Table 4).

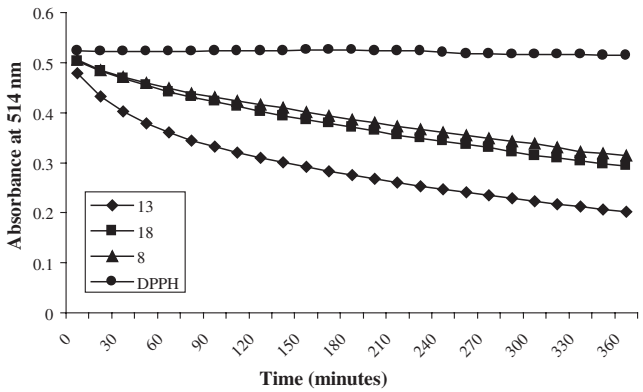


Figure 1. Time courses of decrease in DPPH concentration for compound **8**, **13** and **18**.

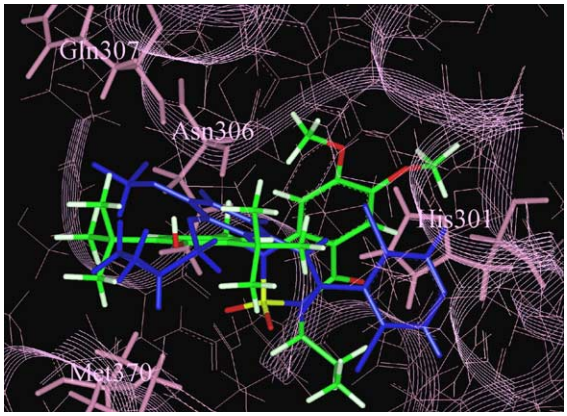


Figure 2. Overlay of the docked orientations for roflumilast (blue) and compound **13** in the active site of PDE4D. The most significant amino acid residues are reported and labelled accordingly.

The 6,7-dimethoxy substituted derivative **13**, the most significative as PDE4 inhibitor, showed the best antioxidant property. On the basis of these experimental data, we can deduce that the 3,5-di-*tert*-butyl-4-hydroxybenzyl moiety at N1 produces anti-PDE4 compounds characterized by antioxidant properties.

It is worth mentioning that the combination of PDE4 inhibition and radical scavenging activity in a single compound could prove an efficient strategy for the treatment of chronic inflammatory diseases by a double and synergic mechanism of action.

4. Experimental

4.1. Chemistry

Melting points were determined in capillary tubes (Büchi 510 capillary apparatus) and are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Brucker DPX 200 spectrometer using DMSO-d_6 as solvent. Chemical shifts were reported in δ (ppm) units relative to internal reference tetramethylsilane (TMS). Coupling constants (J) values were given in Hertz. Multiplicities are abbreviated as follows: s, singlet; d, doublet; t, triplet; dd, double doublet; ddd, double double doublet; dt, double triplet; sxt, sextet; sep, septet; b, indicates a broadening of the signal; *, D_2O changeable. IR spectra were recorded on a Perkin–Elmer 1600 FT-IR spectrometer (Nujol mull) and UV spectra on a Cary 50 Bio UV–VISIBLE Spectrophotometer Varian. Mass spectra were obtained using a Finnigan Mat SSQ710A spectrometer. Thin layer chromatography (TLC) performed on aluminium silica gel sheets 60 F_{254} was used for confirming the purity on analytical samples. Flash chromatography was performed on Silica gel Merck (230–400 mesh). Elemental analyses for C, H, N were performed by a Carlo Erba Elemental Analyzer 1106 apparatus. Reagents and solvents were purchased from common commercial suppliers.

4.1.1. General procedure for the synthesis of 3-substituted 2,1,3-BTDs (1–6). To a stirred and cooled (0 °C) solution

of appropriate amine (85 mmol) in dichloromethane (50 mL), chlorosulfonic acid (3.30 g, 28.3 mmol) was added drop by drop and the reaction mixture was stirred for an additional hour at room temperature. After evaporation under reduced pressure, the resultant salt between sulfamic acid and the corresponding amine was dissolved in toluene (50 mL) and treated with phosphorus pentachloride (7.06 g, 33.9 mmol). The mixture was refluxed for 1 h and the inorganic by products removed by filtration. The filtrate was evaporated under reduced pressure to give the sulfamoyl chloride as an oily residue that was used in the next synthetic step without further purification.

To a solution of suitable 2-aminobenzoate (18.32 mmol) and triethylamine (2.73 g, 27 mmol) in toluene (50 mL) was slowly added a solution of appropriate sulfamoyl chloride (21.70 mmol) in toluene (10 mL) and the resulting mixture was heated at 80 °C for 1 h. The triethylamine hydrochloride was filtered and the filtrate was evaporated under reduced pressure to give an oily residue. The residue was dissolved in 75 mL of a 0.5 M sodium methoxide methanolic solution, freshly prepared. After stirring for 2 h at 40 °C, the solvent was evaporated under reduced pressure and the residue dissolved in water. After cooling and acidification with hydrochloric acid 6 N, the aqueous solution supplied **1**–**6** derivatives as a solid residue.

The solid was collected by filtration and recrystallized from proper solvent(s).

4.1.1.1. 3-Propyl-6,7-dimethoxy-1*H*-2,1,3-benzothiadiazin-4(3*H*)-one 2,2-dioxide (2). Yield 1.38 g (25%), mp 151–152 °C (DMF/H_2O); ^{1}H NMR: δ 7.40 (1H, s, aromatic H-5), 6.70 (1H, s, aromatic H-8), 3.85 (3H, s, OCH_3), 3.80 (3H, s, OCH_3), 3.77 (2H, t, J = 7.4 Hz, NCH_2), 1.67 (2H, sxt, J = 7.4 Hz, CH_2), 0.89 (3H, t, J = 7.4 Hz, CH_3). IR: ν_{max} (cm^{-1}) 3130, 1731, 1022, 985, 791. Anal. Calcd for $C_{12}H_{16}N_2O_5S$: C, 47.99; H, 5.37; N, 9.33. Found: C, 48.13; H, 5.45; N, 9.61.

4.1.1.2. 3-Benzyl-7-chloro-1*H*-2,1,3-benzothiadiazin-4(3*H*)-one 2,2-dioxide (4). Yield 0.89 g (15%), mp 192–193 °C (CH_3OH/H_2O); ^{1}H NMR: δ 7.90 (1H, d, J = 8.5 Hz, H-5), 7.26 (7H, m, aromatic H), 4.97 (2H, s, NCH_2Ph). IR: ν_{max} (cm^{-1}) 3172, 1650, 1351, 1176, 720, 700. Anal. Calcd for $C_{14}H_{11}ClN_2O_3S$: C, 52.10; H, 3.44; N, 8.68. Found: C, 52.49; H, 3.83; N, 8.92.

4.1.1.3. 3-Benzyl-6,7-dimethoxy-1*H*-2,1,3-benzothiadiazin-4(3*H*)-one 2,2-dioxide (5). Yield 5.74 g (90%), mp 160–161 °C (CH_3OH/H_2O); ^{1}H NMR: δ 7.40 (1H, s, H-5) 7.32 (5H, m, aromatic H), 6.72 (1H, s, H-8), 5.00 (2H, s, NCH_2Ph), 3.86 (3H, s, OCH_3), 3.80 (3H, s, OCH_3). IR: ν_{max} (cm^{-1}) 3170, 1673, 1277, 1165, 1004, 722. Anal. Calcd for $C_{16}H_{16}N_2O_5S$: C, 55.16; H, 4.63; N, 8.04. Found: C, 54.88; H, 4.63; N, 8.43.

The known 3-isopropyl-1*H*-2,1,3-benzothiadiazin-4(3*H*)-one 2,2-dioxide (Bentazon)[30] (**1**), 3-benzyl-1*H*-2,1,3-benzothiadiazin-4(3*H*)-one 2,2-dioxide (**3**)[31] and 3-(2-phenylethyl)-1*H*-2,1,3-benzothiadiazin-4(3*H*)-one 2,2-dioxide (**6**)[32] were prepared using the procedure described above.

4.1.2. General procedure for the synthesis of 1,3-disubstituted 2,1,3-BTDs (7–19). The suitable 3-substituted 1*H*-2,1,3-benzothiadiazin-4(3*H*)one 2,2-dioxide (4.16 mmol) and triphenylphosphine (TPP) (1.48 g, 5.64 mmol) were added to a solution of the proper alcohol (2.82 mmol) in anhydrous tetrahydrofuran (THF) (10 mL). Over a period of 5 min, diisopropylazodicarboxylate (DIAD) (1.14 g, 5.64 mmol) was added: the orange-red colour of DIAD disappears immediately with slight liberation of heat. The mixture was stirred at room temperature overnight in N_2 atmosphere. The solvent was evaporated under reduced pressure and the residue was purified by flash column chromatography (cyclohexane/ethyl acetate 5/5, **7**; cyclohexane/ethyl acetate 6/4, **8**; chloroform/acetone 9.5/0.5, **9**, **10**, **14**, **15**; chloroform/acetone 9.8/0.2, **12**, **13**, **18**; chloroform **19**) and recrystallized from proper solvent(s).

4.1.2.1. 1-(4-Hydroxybenzyl)-3-isopropyl-1*H*-2,1,3-benzothiadiazin-4(3*H*)-one 2,2-dioxide (7). Yield 0.20 g (21%), mp 133 °C (CH_3OH/H_2O); ^{1}H NMR: δ 9.51 (1H, s*, OH), 7.98 (1H, dd, J = 1.6, 7.8 Hz, H-5), 7.78 (1H, ddd, J = 1.6, 7.5, 8.2 Hz, H-7), 7.60 (1H, dd, J = 1.1, 8.2 Hz, H-8), 7.46 (1H, ddd, J = 1.1, 7.5, 7.8 Hz, H-6), 6.90 (2H, m, *meta* phenolic), 6.64 (2H, m, *ortho* phenolic), 4.99 (2H, s, NCH_2Ph), 4.77 (1H, sep, J = 6.9 Hz, CH), 1.38 (6H, d, J = 6.9 Hz, CH_3). IR: ν_{max} (cm^{-1}) 3284, 1651, 1379, 1199, 1031. Anal. Calcd for $C_{17}H_{18}N_2O_4S$: C, 58.95; H, 5.24; N, 8.09. Found: C, 58.96; H, 5.46; N, 8.23.

4.1.2.2. 1-(3,5-Di-*tert*-butyl-4-hydroxybenzyl)-3-isopropyl-1*H*-2,1,3-benzothiadiazin-4(3*H*)-one 2,2-dioxide (8). Yield 0.58 g (45%), mp 97–100 °C (CH_3OH/H_2O); ^{1}H NMR: 8.01 (1H, dd, J = 1.6, 7.9 Hz, H-5), 7.78 (1H, ddd, J = 1.6, 7.5, 8.2 Hz, H-7), 7.65 (1H, bd, J = 8.2 Hz, H-8), 7.48 (1H, bdd, J = 7.5, 7.9 Hz, H-6), 6.92 (1H, s*, OH), 6.67 (2H, s, aromatic H), 4.96 (2H, s, NCH_2Ph), 4.54 (1H, sep, J = 6.9 Hz, CH), 1.20 (18H, s, CH_3 *tert*-but.), 1.15 (6H, d, J = 6.9 Hz, CH_3). IR: ν_{max} (cm^{-1}) 3589, 1670, 1602, 1310, 1194, 1116. Anal. Calcd for $C_{25}H_{34}N_2O_4S$: C, 65.47; H, 7.47; N, 6.11. Found: C, 65.46; H, 7.54; N, 6.37.

4.1.2.3. 1-(3-Nitrobenzyl)-3-isopropyl-1*H*-2,1,3-benzothiadiazin-4(3*H*)-one 2,2-dioxide (9). Yield 0.87 g (82%), mp 137–140 °C (trituration with petrol ether 40–60 °C); ^{1}H NMR: δ 8.16 (1H, m, aromatic H), 8.05 (1H, pseudo s, aromatic H), 8.01 (1H, dd, J = 1.7, 7.9 Hz, H-5), 7.76 (1H, ddd, J = 1.7, 7.5, 8.1 Hz, H-7), 7.55 (3H, m, aromatic H), 7.45 (1H, ddd, J = 1.1, 7.5, 7.9 Hz, H-6), 5.28 (2H, s, NCH_2Ph), 4.82 (1H, sep, J = 6.9 Hz, CH), 1.38 (6H, d, J = 6.9 Hz, CH_3). IR: ν_{max} (cm^{-1}) 1681, 1531, 1195, 1174, 977. Anal. Calcd for $C_{17}H_{17}N_2O_5S$: C, 54.39; H, 4.56; N, 11.19. Found: C, 54.11; H, 4.18; N, 11.27.

4.1.2.4. 1-Cyclohexyl-3-isopropyl-1*H*-2,1,3-benzothiadiazin-4(3*H*)-one 2,2-dioxide (10). Yield 0.11 g (12%), mp 80–84 °C (trituration with petrol ether 40–60 °C);

^{1}H NMR: δ 8.02 (1H, dd, J = 1.7, 7.9 Hz, H-5), 7.76 (1H, ddd, J = 1.7, 7.5, 8.1 Hz, H-7), 7.55 (1H, ddd, J = 1.2, 7.5, 7.9 Hz, H-6), 7.52 (1H, dd, J = 1.2, 8.1 Hz, H-8), 4.88 (1H, sep, J = 6.9 Hz, CH), 3.98 (1H, m, CH cyclohexyl), 1.51 (10H, m, cyclohexyl), 1.46 (6H, d, J = 6.9 Hz, CH_3). IR: ν_{max} (cm^{-1}) 1668, 1596, 1298, 1193, 985. Anal. Calcd for $C_{16}H_{22}N_2O_3S$: C, 59.60; H, 6.88; N, 8.69. Found: C, 60.00; H, 6.55; N, 8.95.

4.1.2.5. 1-[2-(3-Isopropyl-2,2-dioxido-4-oxo-3,4-dihydro-1*H*-2,1,3-benzothiadiazin-1-yl)ethyl]pyrrolydine-2,5-dione (12). Yield 0.20 g (20%), mp 135 °C (trituration with petrol ether 40–60 °C); ^{1}H NMR: δ 8.05 (1H, dd, J = 1.5, 8.0 Hz, H-5), 7.78 (1H, dt, J = 1.5, 8.1 Hz, H-7), 7.54 (1H, bd, J = 8.1 Hz, H-8), 7.43 (1H, bt, J = 8.1 Hz, H-6), 4.86 (1H, sep, J = 7.0 Hz, CH), 4.07 (2H, m, NCH_2–CH_2N), 3.66 (2H, m, NCH_2CH_2N), 2.46 (4H, m, CH_2), 1.47 (6H, d, J = 7.0 Hz, CH_3). IR: ν_{max} (cm^{-1}) 1704, 1681, 1604, 1168, 757. Anal. Calcd for $C_{16}H_{19}N_3O_5S$: C, 52.59; H, 5.24; N, 11.50. Found: C, 52.73; H, 5.24; N, 11.46.

4.1.2.6. 1-(3,5-Di-*tert*-butyl-4-hydroxybenzyl)-3-propyl-6,7-dimetoxy-1*H*-2,1,3-benzothiadiazin-4(3*H*)-one 2,2-dioxide (13). Yield 0.83 g (57%), mp 163–165 °C (CH_3OH/H_2O); ^{1}H NMR: δ 7.34 (1H, s, aromatic H-5), 7.17 (1H, s, aromatic H-8), 6.94 (1H, s*, OH), 6.68 (2H, s, aromatic H), 4.87 (2H, s, NCH_2Ph), 3.91 (3H, s, OCH_3), 3.83 (3H, s, OCH_3), 3.46 (2H, t, J = 7.3 Hz, NCH_2), 1.49 (2H, sxt, J = 7.3 Hz, CH_2), 1.24 (18H, s, *tert*-butyl), 0.82 (3H, t, J = 7.3 Hz, CH_3). IR: ν_{max} (cm^{-1}) 3615, 1672, 1606, 1263, 1181, 1011. Anal. Calcd for $C_{27}H_{38}N_2O_6S$: C, 62.52; H, 7.38; N, 5.40. Found: C, 62.47; H, 7.46; N, 5.80.

4.1.2.7. 3-Benzyl-1-(4-hydroxybenzyl)-1*H*-2,1,3-benzothiadiazin-4(3*H*)-one 2,2-dioxide (14). Yield 0.38 g (34%), mp 98 °C; ^{1}H NMR: δ 9.44 (1H, s*, OH), 7.97 (1H, dd, J = 1.7, 7.7 Hz, H-5), 7.78 (1H, dt, J = 1.7, 7.7 Hz, H-7), 7.61 (1H, dd, J = 1.2, 7.7 Hz, H-8), 7.45 (1H, dt, J = 1.2, 7.7 Hz, H-6), 7.33 (5H, m, aromatic H), 6.82 (2H, m, *meta* phenolic), 6.56 (2H, m, *ortho* phenolic), 4.97 (2H, s, NCH_2), 4.93 (2H, s, NCH_2). IR: ν_{max} (cm^{-1}) 3296, 1650, 1461, 1183, 1024, 629. Anal. Calcd for $C_{21}H_{18}N_2O_4S$: C, 63.95; H, 4.60; N, 7.10. Found: C, 64.05; H, 4.88; N, 7.41.

4.1.2.8. 3-Benzyl-1-(3-nitrobenzyl)-1*H*-2,1,3-benzothiadiazin-4(3*H*)-one 2,2-dioxide (15). Yield 0.76 g (64%), mp 108–109 °C (CH_3OH); ^{1}H NMR: δ 8.14 (1H, m, aromatic H), 8.04 (2H, dd, J = 1.6, 7.8 Hz, aromatic H), 7.80 (1H, ddd, J = 1.6, 7.4, 8.0 Hz), 7.58 (3H, m, aromatic H), 7.49 (1H, ddd, J = 1.0, 7.6, 8.0 Hz), 7.33 (5H, m, aromatic H), 5.28 (2H, s, NCH_2Ph), 4.99 (2H, s, NCH_2Ph). IR: ν_{max} (cm^{-1}) 1688, 1601, 1526, 1180. Anal. Calcd for $C_{21}H_{17}N_3O_5S$: C, 59.57; H, 4.05; N, 9.92. Found: C, 60.19; H, 3.79; N, 10.15.

4.1.2.9. 3-Benzyl-1-(3,5-di-*tert*-butyl-4-hydroxybenzyl)-7-chloro-1*H*-2,1,3-benzothiadiazin-4(3*H*)-one 2,2-dioxide (18). Yield 0.55 g (36%), mp 115–119 °C (CH_3OH/H_2O); ^{1}H NMR: δ 7.98 (1H, d, J = 8.5 Hz, aromatic H-5), 7.91 (1H, d, J = 2.0 Hz, aromatic H-8), 7.59 (1H, dd, J = 8.5 Hz, J = 2.0 Hz, aromatic H-6), 7.33 (5H, m, aromatic H), 7.04 (1H, s*, OH), 6.78 (2H, s, aromatic H), 5.03 (2H, s, NCH_2Ph), 4.70 (2H, s, NCH_2Ph), 1.27 (18H, s, CH_3). IR: ν_{max} (cm^{-1}) 3575, 1683, 1597, 1292, 1174, 1115. Anal. Calcd for $C_{29}H_{33}ClN_2O_4S$: C, 64.37; H, 6.15; N, 5.18. Found: C, 64.51; H, 6.35; N, 5.54.

4.1.2.10. 1-(2-Nitrobenzyl)-3-(2-phenylethyl)-1*H*-2,1,3-benzothiadiazin-4(3*H*)-one 2,2-dioxide (19). Yield 0.91 g (74%), mp 112–115 °C (trituration with petroleum ether 40–60 °C); ^{1}H NMR: δ 8.09 (2H, dt, J = 1.5, 8.2 Hz, aromatic H), 7.76 (1H, dt, J = 1.5, 7.6 Hz, aromatic H), 7.65 (2H, m, aromatic H), 7.47 (2H, m, aromatic H), 7.26 (6H, m, aromatic H), 5.33 (2H, s, NCH_2Ph), 4.06 (2H, m, NCH_2CH_2Ph), 2.93 (2H, m, NCH_2CH_2Ph). IR: ν_{max} (cm^{-1}) 1682, 1601, 1537, 1262, 1147. Anal. Calcd for $C_{22}H_{19}N_3O_5S$: C, 60.40; H, 4.38; N, 9.61. Found: C, 60.78; H, 4.40; N, 9.99.

The known 2-[(3-isopropyl-2,2-dioxido-4-oxo-3,4-dihydro-1*H*-2,1,3-benzothiadiazin-1-yl)methyl]-1*H*-isoindole-1,3(2*H*)dione (**11**),[33] 3-benzyl-1-(2-furylmethyl)-1*H*-2,1,3-benzothidiazin-4(3*H*)-one 2,2-dioxide (**16**),[32] 2-[(3-benzyl-2,2-dioxido-4-oxo-3,4-dihydro-1*H*-2,1,3-benzothiadiazin-1-yl)methyl]-1*H*-isoindole-1,3(2*H*)dione (**17**)[32] were prepared using the procedure described above.

4.1.2.11. 6-Chloro-3-chloromethyl-3,4-dihydro-2*H*-1,2,4-benzothiadiazin 1,1-dioxide (26). To a solution of 2-amino-4-chlorobenzensulfonamide (10 g, 48 mmol) in DMF (50 mL) was added a solution of 45% aqueous chloroacetaldehyde (16.9 g, 96 mmol) and NH_4Cl (3.0 g, 58 mmol) in water (10 mL). The reaction mixture was heated for 1 h at 100 °C, cooled and poured into water to obtain **26** as a beige solid. Yield 12.18 g (95%) mp 173 °C (DMF/water); ^{1}H NMR: δ 7.83 (1H, d*, J = 11.2 Hz, NH-4), 7.52 (1H, s*, NH-2), 7.50 (1H, d, J = 8.4 Hz, H-8), 6.92 (1H, d, J = 2.0 Hz, H-5), 6.77 (1H, dd, J = 2.0, 8.4 Hz, H-7), 4.92 (1H, m, H-3), 3.81 (2H, d, J = 7.6 Hz, CH_2Cl). IR: ν_{max} (cm^{-1}) 3359, 3218, 1603, 1166, 765. Anal. Calcd for $C_8H_8Cl_2N_2O_2S$: C, 35.97; H, 3.02; N, 10.49. Found: C, 36.00; H, 3.12; N, 10.56.

4.1.3. General procedure for the synthesis of 2-substituted 1,2,4-BTDs (20–25). The title compounds **20**–**25** were obtained following the same procedure described for **7**–**19** by reaction of 6-chloro-3-chloromethyl-3,4-dihydro-2*H*-1,2,4-benzothiadiazine 1,1-dioxide **26** with the appropriate alcohol. The residue was purified by flash column chromatography (cyclohexane/ethyl acetate 6.5/3.5, **20**; chloroform/acetone 8/2, **21**; chloroform, **22**, **23**; chloroform/acetone 9.8/0.2, **24**; cyclohexane/ethyl acetate 9/1, **25**).

4.1.3.1. 6-Chloro-3-chloromethyl-2-(4-hydroxybenzyl)-3,4-dihydro-2*H*-1,2,4-benzothiadiazin 1,1-dioxide (20). Yield 0.91 g (86%), mp 156–158 °C; ^{1}H NMR: δ 9,34 (1H, s*, OH), 7,68 (1H, d*, J = 2,0 Hz, NH), 7.57 (1H, d, J = 8,5 Hz, aromatic H-8), 7.16 (2H, m, *meta* phenolic), 6.97 (1H, d, J = 2.0 Hz, aromatic H-5), 6.84 (1H, dd, J = 8.5, 2.0 Hz, aromatic H-7), 6.70 (2H, m, *ortho*

phenolic), 5.10 (1H, bt, $J = 6.8$ Hz, H-3), 4.17 (1H, d, $J = 17.5$ Hz, NCH*H*Ph), 3.86 (3H, m, CH_2Cl and NCH*H*Ph). IR: ν_{max} (cm^{-1}) 3249, 1737, 1688, 1599, 1526, 1260, 1110, 1051, 926. Anal. Calcd for $C_{15}H_{14}Cl_2N_2O_3S$: C, 48.27; H, 3.78; N, 7.51. Found: C, 48.14; H, 3.69; N, 7.39.

4.1.3.2. 6-Chloro-3-chloromethyl-2-(pyridin-4-ylmethyl)-3,4-dihydro-2*H*-1,2,4-benzothiadiazin 1,1-dioxide (21). Yield 0.66 g (65%), mp 121–123 °C; ^{1}H NMR: δ 8.51 (2H, bd, $J = 6.0$ Hz, pyridine H-2 and H-6), 7.80 (1H, d*, $J = 2.0$ Hz, NH), 7.58 (1H, d, $J = 8.5$Hz, aromatic H-8), 7.42 (2H, bd, $J = 6.0$ Hz, pyridine H-3 and H-5), 7.02 (1H, d, $J = 2.0$ Hz, aromatic H-5), 6.87 (1H, dd, $J = 2.0$, 8.5 Hz, aromatic H-7), 5.32 (1H, m, H-3), 4.39 (1H, d, $J = 17.0$ Hz, NCH*H*Ph), 3.96 (3H, m, CH_2Cl and NCH*H*Ph). IR: ν_{max} (cm^{-1}) 3370, 1704, 1697, 1599, 1337, 1167, 1080, 722. Anal. Calcd for $C_{14}H_{13}Cl_2N_3O_2S$: C, 46.94; H, 3.66; N, 11.73. Found: C, 47.05; H, 3.99; N, 12.00.

4.1.3.3. 6-Chloro-3-chloromethyl-2-(2-nitrobenzyl)-3,4-dihydro-2*H*-1,2,4-benzothiadiazin 1,1-dioxide (22). Yield 0.42 g (37%), mp 144–145 °C; ^{1}H NMR: δ 8.06 (H, d, $J = 8.1$ Hz, nitrobenzyl aromatic H-3), 8.02 (H, d, $J = 7.8$ Hz, nitrobenzyl aromatic H-6), 7.86 (1H, d*, $J = 2.0$ Hz, NH), 7.82 (1H, dd, $J = 7.8$, 8.6 Hz, nitrobenzyl aromatic H-5), 7.59 (1H, d, $J = 8.5$ Hz, aromatic H-8), 7.57 (1H, dd, $J = 8.1$, 8.6 Hz, nitrobenzyl aromatic H-4), 7.06 (1H, d, $J = 2.0$ Hz, aromatic H-5), 6.89 (1H, dd, $J = 2.0$, 8.5 Hz, aromatic H-7), 5.34 (1H, ddd, $J = 2.0$, 5.7, 7.9 Hz, H-3), 4.70 (1H, d, $J = 17.5$ Hz, NCH*H*Ph), 4.36 (1H, d, $J = 17.5$ Hz, NCH*H*Ph), 4.06 (1H, dd, $J = 7.9$, 11.7 Hz, CH*H*Cl), 3.87 (1H, dd, $J = 5.7$, 11.7 Hz, CH*H*Cl). IR: ν_{max} (cm^{-1}) 3371, 1595, 1561, 1321, 1160, 1093, 859, 727. Anal. Calcd for $C_{15}H_{13}Cl_2N_3O_4S$: C, 44.79; H, 3.26; N, 10.45. Found: C, 45.11; H, 3.35; N, 10.57.

4.1.3.4. 6-Chloro-3-chloromethyl-2-(3-nitrobenzyl)-3,4-dihydro-2*H*-1,2,4-benzothiadiazin 1,1-dioxide (23). Yield 0.79 g (70%), mp 163–165 °C; ^{1}H NMR: δ 8.30 (1H, bs, nitrobenzyl aromatic H-2), 8.12 (1H, bd, $J = 8.3$ Hz, nitrobenzyl aromatic H-4), 7.86 (1H, bd, $J = 7.8$Hz, nitrobenzyl aromatic H-6), 7.79 (1H, d*, $J = 2.0$ Hz NH), 7.66 (1H, dd, $J = 7.8$, 8.3 Hz, nitrobenzyl aromatic H-5), 7.59 (1H, d, $J = 8.5$ Hz, aromatic H-8), 6.98 (1H, d, $J = 2.0$ Hz, aromatic H-5), 6.86 (1H, dd, $J = 2.0$, 8.5 Hz, aromatic H-7), 5.34 (1H, ddd, $J = 2.0$, 5.7, 8.1 Hz, H-3), 4.50 (1H, d, $J = 16$,6 Hz, NCH*H*Ph), 4.16 (1H, d, $J = 16.6$ Hz, NCH*H*Ph), 4.05 (1H, dd, $J = 8.1$, 11.7 Hz, CH*H*Cl), 3.89 (1H, dd, $J = 5.7$, 11.7 Hz, CH*H*Cl). IR: ν_{max} (cm^{-1}) 3385, 1594, 1561, 1526, 1332, 1165, 1088, 726. Anal. Calcd for $C_{15}H_{13}Cl_2N_3O_4S$: C, 44.79; H, 3.26; N, 10.45. Found: C, 44.48; H, 3.37; N, 10.41.

4.1.3.5. 2-{[6-Chloro-3-(chloromethyl)-1,1-dioxido-3,4-dihydro-2*H*-1,2,4-benzothiadiazin-2-yl]methyl}-1*H*-isoindole-1,3-(2*H*)-dione (24). Yield 0.38 g (32%), mp 228–230 °C dec; ^{1}H NMR: δ 7.87 (5H, m, aromatic H and NH), 7.52 (1H, d, $J = 8.5$ Hz, aromatic H-8), 6.85 (1H, d, $J = 2.0$ Hz, aromatic H-5), 6.77 (1H, dd, $J = 2.0$, 8.5 Hz, aromatic H-7), 5.35 (1H, ddd, $J = 3.5$, 6.7, 7.5 Hz, H-3), 5.10 (1H, d, $J = 14.4$ Hz, NCH*H*N), 4.96 (1H, d, $J = 14.4$ Hz, NCH*H*N), 4.05 (1H, dd, $J = 7.5$, 11.2 Hz, CH*H*Cl), 3.87 (1H, dd, $J = 6.7$, 11.2 Hz, CH*H*Cl). IR: ν_{max} (cm^{-1}) 3381, 1777, 1715, 1325, 1130, 881, 718. Anal. Calcd for $C_{17}H_{13}Cl_2N_3O_4S$: C, 47.90; H, 3.07; N, 9.86. Found: C, 48.30; H, 2.91; N, 10.12.

4.1.3.6. 2-{[6-Chloro-3-(chloromethyl)-1,1-dioxido-3,4-dihydro-2*H*-1,2,4-benzothiadiazin-2-yl]ethyl}-pyrrolidine-2,5-dione (25). Yield 0.4 g (36%), mp 139–142 °C; ^{1}H NMR: δ 7.74 (1H, d*, $J = 2.1$ Hz, NH), 7.55 (1H, d, $J = 8.5$ Hz, aromatic H-8), 6.96 (1H, d, $J = 2.0$ Hz, aromatic H-5), 6.84 (1H, dd, $J = 2.0$, 8.5 Hz, aromatic H-7), 5.23 (1H, ddd, $J = 2.1$, 6.5, 6.7 Hz, H-3), 3.94 (2H, m, CH_2Cl), 3.65–2.75 (4H, m, NCH_2CH_2N), 2.58 (4H, s, pyrrolidine). IR: ν_{max} (cm^{-1}) 3330, 1693, 1594, 1164, 721. Anal. Calcd for $C_{14}H_{15}Cl_2N_3O_4S$: C, 42.87; H, 3.85; N, 10.71. Found: C, 43.16; H, 3.96; N, 10.02.

4.2. Biological assays

4.2.1. Chemicals. Benzamidine, bovine serum albumine (BSA, fraction V powder), cAMP, EGTA (ethyleneglycol-bis-[β-amino-ethylether]-*N*,*N*,*N*′,*N*′-tetraacetic acid), 3-isobutyl-1-methyl-xanthine (IBMX), leupeptin, β-mercaptoethanol, pepstatin A and trysin inhibitor were purchased from Sigma Chemie (Deisenhofen, Germany). [5′,8-^{3}H]cAMP and phosphodiesterase SPA assay beads were obtained from Amersham Biosciences (Freiburg, Germany). Pefablock^R SC was purchased from Boehringer Mannheim (Germany). Dimethyl sulfoxide (DMSO) and tris(hydroxymethyl)-aminoethan (Tris) were obtained either from Merck (Darmstadt, Germany) or from Sigma Chemie. All other chemicals were of analytical grade and were obtained from Merck.

For the inhibition experiments (see below), stock solutions (10 mM) of the compounds were prepared in DMSO, which were serially diluted 1:10 (v/v) in DMSO to achieve the desired final concentrations of the compounds in the assays after pipetting of 1 μL of these dilutions into the 100 μL-assays (representing a final dilution step of 1:100 v/v).

4.2.2. PDE enzymes. PDE3 was analyzed using cytosol from human platelets; the homogenate was prepared in analogy to the method described below for insect cells. The human PDE4D3 (GB no. U50159) was a gift of Prof. Marco Conti (Stanford University, USA); the ORF (GB no. U50159) was cut from the original pCMV5 vector with the restriction enzymes EcoRI and XbaI and subcloned in the expression vector pBP9. The human PDE7A1 (GB no. L12052) was isolated using RT-PCR, from total cellular RNA derived from the T-cell line CCRF-CEM using the primers CP2PD7S (5′-GGGCGGGCGGATCCAATGGAAGTG-3′) and CP3PD7A (5′-CTGGTTCTGGGGGTTATGATAACCG-3′) and cloned into the Baculovirus expression vector pCRBac. Recombinant PDEs were expressed in a Baculovirus/Sf21 insect cell system. After infection (usually for 48 h) the Sf21 cells were resus-

pended in ice-cold (4 °C) homogenization buffer (20 mM Tris, pH 8.2, containing the following additions: 140 mM NaCl, 3.8 mM KCl, 1 mM EGTA, 1 mM $MgCl_2$, 10 mM β-mercaptoethanol, 2 mM benzamidine, 0.4 mM Pefablock, 10 μM leupeptin, 10 μM pepstatin A and 5 μM trypsin inhibitor) at a concentration of approximately 10^7 cells/mL, and disrupted by ultrasonication on ice. The homogenate was then centrifuged for 10 min at 1.000*g* (4 °C) and the supernatant was stored at −80 °C until subsequent use.

4.2.3. Measurement of PDE activity. PDE activity was measured by a modified SPA (scintillation proximity assay) test from Amersham Biosciences (see procedural instructions 'phosphodiesterase [^{3}H]cAMP SPA enzyme assay, code TRKQ 7090'), carried out in 96 well microplates (MTPs). The test volume is 100 μL and contains 20 mM Tris buffer (pH 7.4), 0.1 mg/mL BSA, 5 mM Mg^{2+}, 0.5 μM of the substrate cAMP (including about 50.000 cpm of the corresponding [^{3}H]-labelled cyclic nucleotide), 1 μL of the respective substance dilution in DMSO and sufficient enzyme to ensure that 10–20% of the substrate is converted. The final concentration of DMSO in the assays (1% v/v) does not substantially affect any of the PDEs investigated.

After a preincubation of 5 min at 37 °C in the presence of the compounds, the reaction is started by adding the substrate and the assays are incubated for a further 15 min; after that, they are stopped by adding SPA beads (50 μL). In accordance with the manufacturer's instructions, the SPA beads had previously been resuspended in water, but were then further diluted 1:3 (v/v) in water; the diluted solution also contains 3 mM IBMX to ensure a complete PDE activity stop. After the beads have been sedimented (>30 min), the MTPs are analyzed in commercially available luminescence detection devices. IC_{50} values were calculated from the concentration–inhibition-curves by nonlinear regression analysis using GraphPad Prism.

4.3. Radical scavenging effect on DPPH radical

Two millilitres of an ethanolic solution (1×10^{-4} M) of the tested compound was added to 2 mL of a DPPH solution (1×10^{-4} M), and the reaction mixture was shaken vigorously and kept at 37 °C ± 0.02 (Haake F 3C Thermocriostat) in air. DPPH absorption was measured at 514 nm every 15 min. The mean values were obtained from quadruplicate experiments.

4.4. Molecular modelling

Molecular structures of ligands **8**, **13**, **18** were built and energy minimized within MacroModel.[34] Conformational analysis was carried out using the AMBER* force field, as included in MacroModel. For all compounds, the resulting geometries of the lower energy conformers were re-optimized with semi-empirical quantum mechanic calculations, using the Hamiltonian AM1 as implemented in Spartan[35] and atomic charges were calculated.

The three-dimensional structure co-ordinate file of PDE4D catalytic domain (PDB entry 1MKD) in complex with zardaverine was obtained from the Protein Data Bank.[36] A two-step docking protocol was employed. In a first phase, each inhibitor was docked into the active site by means of the FlexX module, as implemented in Sybyl v6.8[25] with the macromolecule and the ligands being flexible. Preparation of the protein for FlexX requires definition of the binding pocket in terms of 'interaction points'. In this work the active site was defined as all atoms within a distance of 10 Å from zardaverine. The specific distance was determined in order to ensure a significative portion of the active site for the docking experiments.

Starting from the best-docked geometries, as obtained with FlexX, the second step consisted in a further refinement of the complex performed with QXP.[37] Also the algorithm implemented in the QXP program allows for fully flexibility of the inhibitors and simultaneous flexibility of the active site side chains. Each docking run included 15,000 steps of Monte Carlo perturbation, subsequent fast searching and final energy minimization. The results were evaluated in terms of total estimated binding energy, internal strain energy of the ligand, van der Waals and electrostatic interaction energies.

For verifying the variability of amino acids residues in S1, S2 and S3 sub-pockets of PDE4D catalytic site, amino acids sequences of PDE family members were retrieved from the SWISSPROT database.[27] Multiple sequence alignment of PDE isozymes was performed employing CLUSTALW.[28] All calculations were carried out on a SGI O2 workstations and on a standard personal computer running under Linux.

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