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Original article

Synthesis and *in vitro* antimycobacterial and isocitrate lyase inhibition properties of novel 2-methoxy-2′-hydroxybenzanilides, their thioxo analogues and benzoxazoles

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ABSTRACT

A new series of 2-methoxy-2′-hydroxybenzanilide derivatives and their thioxo analogues have been synthesised and characterised by IR, NMR and elemental analysis. These compounds were investigated for their *in vitro* antimycobacterial activities against *Mycobacterium tuberculosis 331/88*, *Mycobacterium avium 330/88*, *Mycobacterium kansasii 235/80*, clinically isolated *M. kansasii 6509/96* and the ability to act as *in vitro* isocitrate lyase inhibitors. The best ICL inhibitors were two compounds from the thiobenzanilide group (**8f**, **8m**), which exhibited an inhibition potential that was equal to the standard compound, 3-nitropropionic acid. In addition, the best antimycobacterial properties were exhibited by benzanilide derivatives **6h**, **6k** and **6l** with 5-Cl and 4′ or 5′ Cl/Br substitution. For all the thiobenzanilide derivatives tested, two conformers were observed in the NMR spectra, which is most likely due to the hindered rotation of the C–N bond.

1. Introduction

Benzanilide (*N*-phenylbenzamide) derivatives possess a broad spectrum of biological activities. They have been found to exhibit antimalarial [1] and antibacterial properties [2], and the derivatives of *N*-(2-hydroxyphenyl)benzamide have been synthesised and studied for the last few years as the possible metabolites of the antibacterial active benzo[*d*]oxazole derivatives [3–5]. Benzanilides are also well known for their ability to act as potassium channel openers [6,7] and spasmolytic agents [8]. They have shown antiepileptic activity by affecting the central voltage-dependent sodium channels [9]. More recently, some benzanilide derivatives have been reported to inhibit the c-Met tyrosine kinase receptor, which is a potentially important target for the treatment of cancer [10].

Tuberculosis (TB) is one of the most dangerous and the most widespread infectious diseases. This fact is supported by the new WHO report released in 2011, which stated that there were an estimated 8.8 million new cases of TB with 1.45 million being fatal and more than one third of the global population being infected with *Mycobacterium tuberculosis*, the causative agent of TB [11]. Although most of the cases are located in developing countries, TB has become a serious threat to the modern world due to population migration and its increasing resistance to commonly used antituberculotics. Currently, *M. tuberculosis* strains have been found to be resistant to rifampicin and INH (isoniazid), which are first line treatments and the most powerful antituberculotics (MDR–TB, multidrug-resistant TB). In addition, strains resistant to fluoroquinolones and to at least one of the injectable drugs including kanamycin, capreomycin and amikacin have been already described (XDR–TB, extensively drug-resistant TB) [12]. A further health care problem is the coincidence of HIV and TB infection, which is highlighted by the fact that 1.1 million of all TB cases and 0.35 million of the fatal cases involve HIV positive patients [11].

Therefore, there is still an urgent need for new antituberculotics, particularly those with a new mode of action, that would be less toxic and more effective than the current first and second line antituberculotic drugs that could shorten the total duration of the

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treatment, improve the treatment of MDR–TB and XDR–TB, be effective for co-administration with antiviral agents used to treat AIDS and ultimately improve the treatment of latent TB infections, which is crucial for elimination of TB [13,14]. The molecular target for the last mentioned purpose appears to be the isocitrate lyase (ICL) enzyme in the glyoxylate pathway that is well characterised in plants, fungi, most prokaryotes and lower eukaryotes but has not been observed in vertebrates. The glyoxylate shunt converts isocitrate to succinate and glyoxylate, catalysed by ICL, followed by the addition of Acetyl-CoA to glyoxylate to form malate by malate synthase. The glyoxylate shunt allows above mentioned organisms to avoid the carbon dioxide generating steps of the Krebs cycle and enabling them to use carbons from fats to carbohydrate synthesis. This pathway is also upregulated during the latent phase of *M. tuberculosis* where the metabolism differs from the metabolism observed during the growing phase and the bacteria's carbon source is shifted to C_2 substrates generated by β-oxidation of fatty acids [15].

Herein, we present the results from our study of the antimycobacterial properties of 2-methoxybenzanilides and their thioxo analogues.

2. Chemistry

A new series of 2-methoxy-2′-hydroxybenzanilide derivatives (**6**) were prepared by the condensation reaction of an appropriate 2-methoxybenzoyl chloride (**2**) and a substituted 2-aminophenol (**5**) (Scheme 1). The acid chloride was used due to the failure of the direct condensation in the presence of PCl_3 [16] and was prepared by the treatment of acid (**1**) with excess of $SOCl_2$. Most of the aminophenols were not commercially available and were prepared from the corresponding phenols (**3**) by mild nitration followed by reduction by iron in acetic acid [17]. Next, the prepared anilides (**6**) were converted to their thioxo analogues (**8**). The conversion was performed by reaction with P_4S_{10} in pyridine [18]. Due to the presence of the 2′-OH group in the starting anilides (**6**), the synthesis was complicated by formation of a cyclic salt with P_4S_{10}, which was hydrolysed by HCl in chloroform. The substituents on both aromatic parts of the molecule were selected according to their effect on the antimycobacterial activity in a previously studied group of salicylanilides [16].

3. Pharmacology

3.1. In vitro antimycobacterial assay

All 42 newly prepared compounds were tested in the Laboratory for Mycobacterial Diagnostics and TB at the Institute of Public Health in Ostrava for their *in vitro* antimycobacterial activity against *M. tuberculosis 331/88*, *Mycobacterium avium 330/88*, *Mycobacterium kansasii 235/80* and clinically isolated *M. kansasii 6509/96*. Isoniazid (INH), which is a first line antituberculosis drug, was used as a standard. The compound activity against *M. tuberculosis* and *M. avium* was evaluated after 14 and 21 days, and the compound activity against *M. kansasii* was evaluated after 7, 14 and 21 days. The MIC (minimal inhibition concentration) values represent the lowest concentration of the tested compounds at which the inhibition of mycobacterium growth is observed.

3.2. In vitro enzymatic assay

All compounds were tested for their ability to act as *in vitro* inhibitor of mycobacterial enzyme isocitrate lyase. This evaluation was performed at the Department of Biochemical Sciences, Faculty of Pharmacy in Hradec Králové. The results are presented as a percentage of inhibition and compared to standard 3-nitropropionic acid. Ethionamide and isoniazid were employed as negative controls (inhibition 0%).

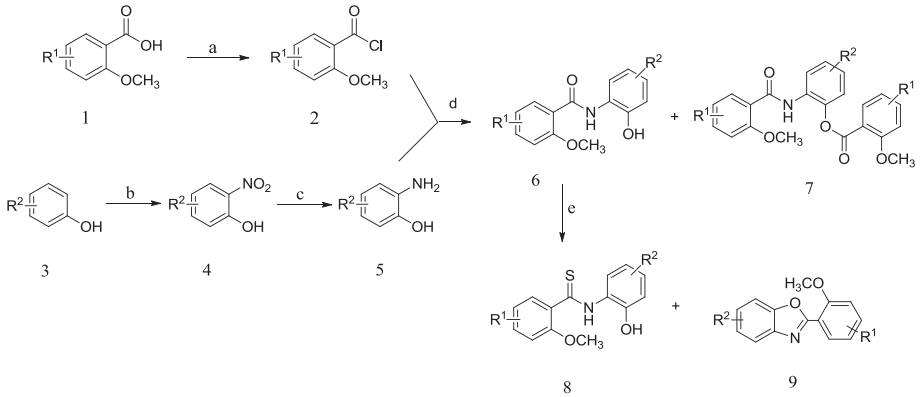
3.3. In vitro cytotoxicity assay of selected compounds

The most active compounds were evaluated for their *in vitro* cytotoxicity. This evaluation was performed at the Department of Pharmacology and Toxicology, Faculty of Pharmacy in Hradec Králové. For this purpose, the human liver cell line Hep G2 and a standard colorimetric test based on determining the reductive metabolic activity of the cells was used. The results are presented as an inhibitory concentration (IC_{50}), which is the concentration of the tested compound required for the reduction of the cell viability value to 50% of the maximum (control) cell viability value. To characterize relation between toxicity and antimycobacterial activity, selectivity index (SI) was calculated. SI value indicates the ratio of IC_{50} and MIC after 14 days of incubation of *M. tuberculosis 331/88*.

4. Results

4.1. Discussion and conclusion

Forty-two antimycobacterial active compounds were synthesised and characterised by ^1H and ^{13}C NMR spectrometry, IR spectrometry, elemental analysis and melting point. The best yields (51–72%) for the 2-methoxy-2′-hydroxybenzanilides (**6**) were obtained when the condensation was performed at room temperature.



Scheme 1. Preparation of 2-methoxy-2′-hydroxybenzanilides (**6**) and their thioxo analogues (**8**) (R^1 = 4-Cl, 5-Cl, R^2 = 3-Cl, 4-Cl 3,4-di-Cl, 3-Br, 4-Br, 3-CF_3, 4-CF_3 – 2-amino-4/5-chlorophenols are available). Reagents and conditions: (a) $SOCl_2$, 110 °C, (b) 65% HNO_3, 99% CH_3COOH, 40 °C, (c) Fe, H_2O, CH_3COOH, 135 °C, (d) TEA, ether, r.t., (e) 1. P_4S_{10}, pyridine, 155 °C, 2. HCl, $CHCl_3$, 110 °C.

The chloride (**2**) was added drop wise in an ether solution with a very low concentration containing an equivalent of triethylamine to a more concentrated ether solution of substituted 2-aminophenol (**5**). Under these conditions, it was possible to reduce the formation of esterified amides (**7**) and the oxidation by-products of the starting aminophenols (**5**). Despite this, four esterified amides were obtained in sufficient amount and purity so could be biologically evaluated. During the preparation of the thioxo analogues (**8**), all of the compounds cyclised to derivatives of 2-(2-methoxyphenyl) benzo[*d*]oxazole (**9**) with yields that were equimolar to the yields of thioamides (22–49%). This cyclisation was also observed on silica gel. Most of these benzoxazoles were obtained in sufficient amount and purity and could also be biologically tested.

The *in vitro* antimycobacterial evaluation of the prepared compounds was used to characterise their ability to act against actively growing mycobacteria under nutrient and oxygen rich conditions. It was revealed that in the group of benzanilides were more active derivatives with 5-Cl than with 4-Cl substitution in the acid portion of the molecule. Further, the substitution of the anilide moiety in position 4 or 5 by Cl or Br was more effective than the substitution with CF_3 and 4,5-di-Cl (Table 1). The activities in the thiobenzanilides group were very similar, and surprisingly, the thioxo group had a negative effect on the activities of the most active anilides (Table 2). However, thioxo group improved the activities of compounds with 4,5-di-Cl and CF_3 substitution in the aniline moiety. All of the thioamides exhibited activities against INH resistant *M. avium* and *M. kansasii* 235/80. The cyclisation to the benzoxazole derivatives **9** negatively affected the antimycobacterial properties (Table 3). Therefore, one possible explanation of low activities of benzoxazole derivatives could be that the mycobacteria cannot cleave the benzoxazole ring to the amide, which is most likely the active form. Table 3 also shows the MIC values of some esterified amides **7**.

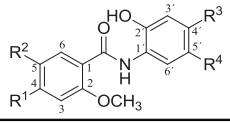
The enzymatic assay of prepared compounds was used to characterised their ability to act as ICL inhibitor and thus potentially against mycobacteria in the latent phase of infection. According to this assay, thioamides **8** were better inhibitors of ICL than amides **6**, and **8f** and **8m** exhibited an activity equivalent to the standard compound, 3-nitropropionic acid (Table 4). Benzoxazole derivatives **9** were found to be inactive, but esterified amides **7** were active (Table 4). Therefore, we can assume that the C=O/S group is essential for inhibitory activity.

The results of the cytotoxicity assay are presented in Table 5. Unfortunately, the great drawback of most of the amides and thioamides is their cytotoxicity. Only two compounds (**6h** and **6k**) were more toxic for *M. tuberculosis* than for human hepatic cell, **6h** with SI after 14 days of incubation 12.8 and **6k** with SI after 14 days of incubation 2.0. Also the best inhibition of ICL (compounds **8f** and **8m**) was observed at a concentration, which was higher than IC_{50} values of these compounds.

For all of the prepared thiobenzanilides (**8**), an interesting result was observed in the proton NMR spectrum, which was measured in DMSO under ambient conditions. Two sets of hydrogen resonance signals were observed with a relative integral intensity ratio of approximately 1:0.15. Contamination of the thioamides (**8**) by the starting benzanilides (**6**) was excluded by comparison of their hydrogen shifts with the hydrogen shifts of the starting benzanilides, whose NMR spectrum was also recorded in DMSO. Further confirmation of the purity was provided by IR spectra and elemental analyses. Based on the coupling constant values, the shape of the peaks and the chemical shift values, the interpretation of both sets of hydrogen signals revealed that each more intense hydrogen signal corresponded to the less intense one. Therefore, there were two OH singlets, two NH singlets, four aromatic doublets of doublets, eight aromatic doublets and two OCH_3 singlets for compound **8i** (Fig. 1). A similar situation was observed in the carbon NMR spectra. However, two complete sets of resonance signals were not always observed due to the low intensity of the less intense signals. Finally, when the proton NMR spectrum of compound **8i** was recorded in DMSO at different temperatures ranging from 25 to 100 °C, each pair of hydrogen resonance signals merged into one signal at 90 °C.

The most likely explanation for this phenomenon is based on the ability of compounds **8** to adopt relatively stable *E* or *Z*

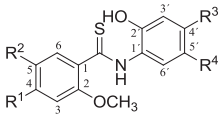
Table 1
Antimycobacterial activities of the prepared 2-methoxy-2′-hydroxybenzanilide derivatives **6a**–**n**.M



	R^1	R^2	R^3	R^4	MIC [μmol L^{-1}]									
					M. tuberculosis My 331/88		*M. avium* My 330/88		*M. kansasii* My 235/80			*M. kansasii* My 6509/96		
					Days									
					14	21	14	21	7	14	21	7	14	21
6a	Cl	H	Cl	H	8	8	62.5	125	62.5	62.5	125	16	62.5	62.5
6b	Cl	H	H	Cl	125	125	125	125	125	125	125	16	62.5	62.5
6c	Cl	H	Cl	Cl	500	500	>1000	>1000	500	500	500	250	250	250
6d	Cl	H	Br	H	125	125	125	125	125	125	125	125	125	125
6e	Cl	H	H	Br	125	125	62.5	62.5	62.5	62.5	62.5	62.5	62.5	62.5
6f	Cl	H	CF_3	H	250	250	62.5	62.5	–[a]	250	250	250	250	250
6g	Cl	H	H	CF_3	250	250	250	250	250	250	250	250	250	250
6h	H	Cl	Cl	H	4	4	62.5	250	16	32	62.5	8	16	16
6i	H	Cl	H	Cl	16	32	125	125	4	16	16	8	16	16
6j	H	Cl	Cl	Cl	125	125	125	125	125	125	125	125	125	125
6k	H	Cl	Br	H	4	8	500	500	500	500	500	250	250	250
6l	H	Cl	H	Br	8	8	62.5	62.5	4	8	8	4	8	8
6m	H	Cl	CF_3	H	500	500	62.5	62.5	–[a]	250	250	250	250	250
6n	H	Cl	H	CF_3	250	250	62.5	125	32	62.5	62.5	62.5	125	125
INH					0.5	0.5	>250	>250	>250	>250	>250	4	4	4

INH – isoniazid.
[a] MIC value was impossible to determine.

Table 2
Antimycobacterial activities of the prepared 2-methoxy-2′-hydroxythiobenzanilide derivatives **8a**–**n**.



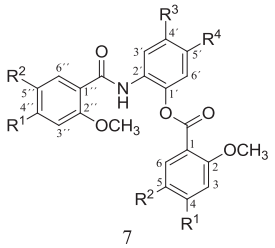
	R^1	R^2	R^3	R^4	MIC [μmol L^{-1}]									
					M. tuberculosis My 331/88		*M. avium* My 330/88		*M. kansasii* My 235/80			*M. kansasii* My 6509/96		
					Days									
					14	21	14	21	7	14	21	7	14	21
8a	Cl	H	Cl	H	16	32	32	32	16	32	62.5	16	32	32
8b	Cl	H	H	Cl	62.5	62.5	32	32	16	62.5	125	32	62.5	125
8c	Cl	H	Cl	Cl	32	32	62.5	62.5	16	32	62.5	16	32	62.5
8d	Cl	H	Br	H	16	32	32	32	16	32	62.5	16	32	32
8e	Cl	H	H	Br	32	32	32	62.5	16	32	62.5	32	32	62.5
8f	Cl	H	CF_3	H	32	62.5	62.5	62.5	–[a]	62.5	62.5	32	62.5	62.5
8g	Cl	H	H	CF_3	32	62.5	32	62.5	32	62.5	125	32	62.5	125
8h	H	Cl	Cl	H	16	32	32	62.5	16	32	62.5	32	32	32
8i	H	Cl	H	Cl	16	32	32	32	8	8	16	16	32	32
8j	H	Cl	Cl	Cl	16	32	62.5	62.5	32	62.5	62.5	16	32	62.5
8k	H	Cl	Br	H	32	32	62.5	125	16	32	62.5	16	32	32
8l	H	Cl	H	Br	16	32	32	32	8	16	16	16	16	32
8m	H	Cl	CF_3	H	62.5	62.5	32	62.5	–[a]	62.5	62.5	32	62.5	62.5
8n	H	Cl	H	CF_3	32	32	62.5	62.5	32	62.5	62.5	16	32	32
INH					0.5	0.5	>250	>250	>250	>250	>250	4	4	4

INH – isoniazid.
[a] MIC value was impossible to determine.

conformation about the CS–NH bond (Fig. 2). These conformers manifested themselves spectroscopically in NMR spectra due to slow rotation on the NMR timescale, but could not be isolated due to fast rotation on the laboratory timescale.

The literature provides much more information on *E*, *Z* conformation of nitrogen containing compounds in the group of benzanilides than in thiobenzanilide group. But to some extent these two groups are similar to each other. Benzanilides are well known for their ability to prefer due to steric reasons either *E* or *Z* conformation about the CO–NH bond [19]. The free rotation about this bond is restricted due to the existence of the rotational barrier, what can make the conformers observable on the NMR timescale.

Table 3
Antimycobacterial activities of isolated esterified amides **7a**–**d** and benzoxazole by-products **9a**–**j**.



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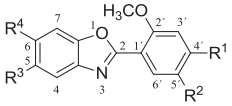

9

	R^1	R^2	R^3	R^4	MIC [μmol L^{-1}]									
					M. tuberculosis My 331/88		*M. avium* My 330/88		*M. kansasii* My 235/80			*M. kansasii* My 6509/96		
					Days									
					14	21	14	21	7	14	21	7	14	21
7a	Cl	H	H	Cl	4	8	62.5	62.5	16	32	125	8	16	62.5
7b	Cl	H	H	CF_3	250	250	62.5	62.5	–[a]	250	250	250	250	250
7c	H	Cl	Cl	Cl	8	8	62.5	62.5	4	8	8	4	8	8
7d	H	Cl	H	CF_3	250	250	62.5	62.5	–[a]	8	16	8	8	8
9a	Cl	H	H	Cl	125	125	62.5	62.5	62.5	62.5	62.5	62.5	62.5	62.5
9b	Cl	H	Cl	Cl	250	250	250	500	250	250	250	250	250	250
9c	Cl	H	Br	H	250	250	250	250	250	250	250	250	250	250
9d	Cl	H	H	Br	125	125	125	125	125	125	125	125	125	125
9e	Cl	H	H	CF_3	250	250	125	125	–[a]	250	250	250	250	250
9f	H	Cl	Cl	H	125	125	250	250	250	250	250	250	250	250
9g	H	Cl	Cl	Cl	125	125	125	125	125	125	125	125	125	125
9h	H	Cl	Br	H	250	250	250	250	250	250	250	250	250	250
9i	H	Cl	H	Br	125	125	125	125	125	125	125	125	125	125
9j	H	Cl	H	CF_3	62.5	62.5	250	250	250	250	250	62.5	62.5	62.5
INH					0.5	0.5	>250	>250	>250	>250	>250	4	4	4

INH – isoniazid.
[a] MIC value was impossible to determine.

Table 4
Inhibition activity of prepared compounds **6**–**9** against isocitrate lyase. Concentration of all the tested and control compounds was 10 μmol L^{-1}.

	% Of inhibition	Standard deviation		% Of inhibition	Standard deviation
6a	4	±3.01	**6h**	10	±0.17
6b	0	0	**6i**	0	0
6c	0	0	**6j**	7	±0.50
6d	0	0	**6k**	0	0
6e	0	0	**6l**	0	0
6f	7	±2.33	**6m**	2	±0.66
6g	0	0	**6n**	0	0
7a	0	0	**7c**	3	±1.44
7b	8	±2.41	**7d**	9	±2.22
8a	10	±2.74	**8h**	0	0
8b	9	±2.28	**8i**	0	0
8c	0	0	**8j**	9	±3.30
8d	10	±1.51	**8k**	7	±1.94
8e	0	0	**8l**	8	±1.37
8f	23	±1.36	**8m**	21	±2.14
8g	0	0	**8n**	8	±4.8
9a	0	0	**9f**	0	0
9b	0	0	**9g**	4	±3.27
9c	0	0	**9h**	0	0
9d	0	0	**9i**	0	0
9e	0	0	**9j**	0	0
3-NPA	25	4.13	**3-NPA**	25	4.13
ETA	0	0	**ETA**	0	0
INH	0	0	**INH**	0	0

3-NPA – 3-nitropropionic acid, ETA – ethionamide, INH – isoniazid.

For the substantial contribution to the rotational barrier is responsible the conjugation of the lone electron pair of nitrogen atom over the carbonyl group and thus, partial double bond character of amide bond [20]. The existence of rotational barrier has been also shown in thioamide group. In thioformamide, it has been calculated that the CS–NH bond is in greater abundance partial double than CO–NH bond of formamide, because of the greater conjugation of the lone electron pair of nitrogen over thiocarbonyl group of thioformamide than over carbonyl group of formamide [21]. This can be the reason that we have been able to observe the two conformers of **8** but not of **6**.

The conformational interconversion of compounds (**8**) could be also slowed down by intramolecular hydrogen bonds. In *Z*-conformer it is possible the existence of three-centre hydrogen bond (Fig. 2), which has been described in similar compound – 2,2′-dimethoxybenzanilide [22]. In this amide, the CONH group hydrogen

Table 5
Cytotoxicity of 2-methoxy-2′-hydroxybenzanilide derivatives **6a**–**n** and 2-methoxy-2′-hydroxythiobenzanilide derivatives **8a**–**n**.

	IC_{50} [μmol L^{-1}]	SI for My 331/88, 14 d		IC_{50} [μmol.L^{-1}]	SI for My 331/88, 14 d
6a	7.34	0.92	**6h**	51.30	12.83
6b	87.02	0.70	**6i**	–[a]	–
6c	11.52	0.02	**6j**	2.98	0.02
6d	46.62	0.37	**6k**	7.98	2.00
6e	5.63	0.05	**6l**	4.09	0.51
6f	3.90	0.02	**6m**	4.91	0.01
6g	25.37	0.10	**6n**	15.43	0.06
8a	5.11	0.32	**8h**	4.45	0.28
8b	8.49	0.14	**8i**	8.60	0.54
8c	3.16	0.10	**8j**	3.72	0.23
8d	3.88	0.24	**8k**	7.60	0.24
8e	6.53	0.20	**8l**	8.74	0.55
8f	3.14	0.10	**8m**	3.69	0.06
8g	5.74	0.18	**8n**	5.86	0.18

[a] IC_{50} value was impossible to determine.

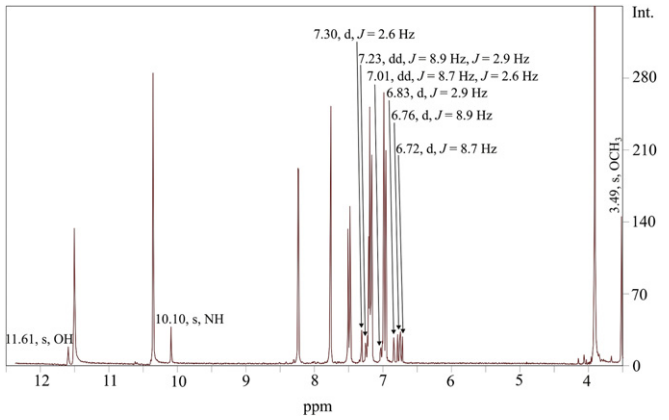

Fig. 1. ^{1}H NMR spectrum of compound **8i** in DMSO at ambient temperature. The relative integral intensity ratio of both conformers 1:0.15. For interpretation of signals of more intense conformer see Experimental protocols.

is involved in both H-bonded five-membered ring and H-bond six-membered ring, resulting in a rigid and planar arrangement of molecule. In addition, the three-centre hydrogen bond acts cooperatively, 5- and 6-membered hydrogen bonding systems enforce each other. The extraordinary stability of this H-bonds system has been demonstrated by the formation of helices based on the *m*-oligomers of the 2,2′-dialkoxybenzanilides [23]. Thus, we can assume that three-centre hydrogen bond in Z-conformation of thioamides (**8**) presented further contribution to the rotational barrier (Fig. 2). This can also explain why we have not observed this phenomenon for the previously studied 2-methoxythiobenzanilide derivatives without 2-OH group in the anilide part of the molecule.

Finally, some contribution to the rotational barrier of compounds **8** could be as well caused by the steric effect of the substituents at the *ortho* positions of the aromatic rings and thus by preference of conformations with minimal steric interactions [19]. Steric effect possesses also sulphur atom itself, because of its greater size and longer C=S bond than C=O bond [24].

Based on this information, we have concluded that this phenomenon and conformation behaviour of compounds **8** requires more in-depth study, and the results of this study will be presented in the near future.

5. Experimental protocols

All of the chemicals and solvents used in this study were purchased from Sigma–Aldrich, Prague, Czech Republic and Penta, Prague, Czech Republic and were used without further purification.

5.1. Chemistry

5.1.1. General methods

The reactions were monitored, and the purity of products was verified by thin layer chromatography in which the plates were coated with 0.2 mm of silica gel 60 F_{254} (Merck KGaA, Darmstadt, Germany) and visualised using UV irradiation (254 and 366 nm).

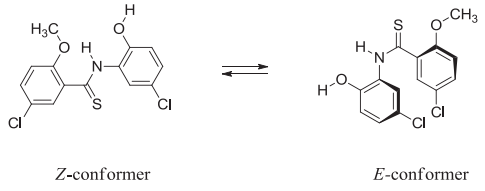

Fig. 2. The possible conformers of thiobenzanilide derivative **8i**.

Column chromatography was performed using silica gel 60 with a particle size of 0.063–0.2 mm (Fluka, Prague, Czech Republic).

The melting points were determined on a Melting Point M-560 apparatus (Bűchi Labortechnik AG, Flawil, Switzerland) in open capillaries and are uncorrected. The IR spectra were recorded on a Nicolet 6700 FT-IR spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) over the range of 400–4000 cm^{-1} using the ATR technique. The NMR spectra were measured in DMSO-d_6 or $CDCl_3$ solutions at ambient temperature on a Varian Mercury Vxbb 300 (300 MHz for ^1H and 75.5 MHz for ^{13}C, Varian Comp. Palo Alto, CA, USA) and Varian Mercury (500 MHz for ^1H and 125 MHz for ^{13}C, Varian Comp. Palo Alto, CA, USA). The chemical shifts (δ) are given in ppm, and tetramethylsilane was employed as the internal standard. Elemental analysis (C, H, N, S) was performed on an automatic microanalyser CHNS-O CE instrument, FISONS EA 1110 (Thermo Fisher Scientific, Waltham, MA, USA).

*5.1.2. General procedure for the preparation of acid chlorides (**2**)*

2.7 mmol of 4/5-chloro-2-methoxybenzoic acid (**1**) was dissolved in 10 mL of $SOCl_2$, and the solution was stirred and refluxed under a $CaCl_2$ drying tube for 3 h. Then, the excess $SOCl_2$ was removed under reduced pressure, and the liquid residue was used without further purification in the next step.

*5.1.3. General procedure for the preparation of substituted 2-methoxy-2′-hydroxybenzanilides (**6**)*

First, 100 mL of an ether solution containing 2.7 mmol of substituted 2-methoxybenzoyl chloride (**2**) and an equimolar amount of TEA was added drop wise at r.t. (1.5 h) into 40 mL of a stirred ether solution containing 2.7 mmol of substituted 2-aminophenol (**5**). After the addition, the mixture was stirred for 1 h at r.t. and then extracted with a 5% solution of HCl (50 mL), with a 5% solution of $NaHCO_3$ (50 mL) and with water (50 mL). The organic phase was dried with Na_2SO_4 and purified using column chromatography and crystallisation.

*5.1.3.1. 4-Chloro-N-(4-chloro-2-hydroxyphenyl)-2-methoxybenzamide (**6a**).* Yield 59.0%, grey solid; m. p. 223–225 °C; IR (ATR): 3315 (ν NH), 3185 (b, ν OH), 1638 (amide I), 1608, 1592 (ν CC aromatic), 1548 (amide II), 1508, 1482, 1460 (ν CC aromatic) cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz): δ 10.82 (1H, bs, OH), 10.43 (1H, s, NH), 8.35 (1H, d, J = 8.7 Hz, H6′), 8.05 (1H, d, J = 8.5 Hz, H6), 7.37 (1H, d, J = 1.9 Hz, H3), 7.20 (1H, dd, J = 8.5 Hz, J = 1.9 Hz, H5), 6.92 (1H, d, J = 2.3 Hz, H3′), 6.87 (1H, dd, J = 8.7 Hz, J = 2.3 Hz, H5′), 4.06 (3H, s, OCH_3); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 161.41, 157.92, 147.60, 138.03, 132.99, 127.17, 126.33, 121.50, 120.81, 120.27, 119.09, 114.47, 113.32, 57.29; Anal. Calcd. for $C_{14}H_{11}Cl_2NO_3$ (312.15): C 53.87, H 3.55, N 4.49, Found: C 53.45, H 3.94, N 4.23.

*5.1.3.2. 4-Chloro-N-(5-chloro-2-hydroxyphenyl)-2-methoxybenzamide (**6b**).* Yield 62.3%, orange solid; m. p. 240–242 °C; IR (ATR): 3294 (ν NH), 3142 (b, ν OH), 1642 (amide I), 1610, 1593 (ν CC aromatic), 1546 (amide II), 1506, 1496, 1480, 1458 (ν CC aromatic) cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz): δ 10.61 (1H, bs, OH), 10.50 (1H, s, NH), 8.42 (1H, d, J = 2.6 Hz, H6′), 8.05 (1H, d, J = 8.4 Hz, H6), 7.37 (1H, d, J = 2.0 Hz, H3), 7.21 (1H, dd, J = 8.4 Hz, J = 2.0 Hz, H5), 6.97 (1H, dd, J = 8.6 Hz, J = 2.6 Hz, H4′), 6.90 (1H, d, J = 8.6 Hz, H3′), 4.06 (3H, s, OCH_3); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 161.62, 157.96, 145.37, 138.23, 133.05, 128.29, 123.35, 122.73, 121.57, 120.07, 119.21, 115.71, 113.37, 57.34; Anal. Calcd. for $C_{14}H_{11}Cl_2NO_3$ (312.15): C 53.87, H 3.55, N 4.49, Found: C 53.82, H 3.86, N 4.32.

*5.1.3.3. 4-Chloro-N-(4,5-dichloro-2-hydroxyphenyl)-2-methoxybenzamide (**6c**).* Yield 54.4%, white solid; m. p. 250–252 °C; IR (ATR): 3302 (ν NH), 3153 (b, ν OH), 1633 (amide I), 1589 (ν CC aromatic), 1544 (amide II), 1497, 1487, 1467 (ν CC aromatic) cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz): δ 11.09 (1H, s, OH), 10.48 (1H, s, NH), 8.56 (1H, s, H6′), 8.03 (1H, d, J = 8.5 Hz, H6), 7.35 (1H, d, J = 2.0 Hz, H3), 7.19 (1H, dd, J = 8.5 Hz, J = 2.0 Hz, H5), 7.03 (1H, s, H3′), 4.05 (3H, s, OCH_3); ^{13}C NMR (DMSO-d_6, 125 MHz): δ 161.64, 157.95, 146.34, 138.36, 133.00, 127.47, 124.71, 121.56, 120.59, 120.28, 119.76, 115.60, 113.33, 57.33; Anal. Calcd. for $C_{14}H_{10}Cl_3NO_3$ (346.59): C 48.52, H 2.91, N 4.04, Found: C 48.65, H 2.97, N 4.00.

*5.1.3.4. N-(4-Bromo-2-hydroxyphenyl)-4-chloro-2-methoxybenzamide (**6d**).* Yield 51.5%, grey solid; m. p. 233–235 °C; IR (ATR): 3316 (ν NH), 3178 (b, ν OH), 1635 (amide I), 1608, 1589 (ν CC aromatic), 1541 (amide II), 1503, 1482, 1458 (ν CC aromatic) cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz): δ 10.78 (1H, s, OH), 10.42 (1H, s, NH), 8.30 (1H, d, J = 8.6 Hz, H6′), 8.04 (1H, d, J = 8.4 Hz, H6), 7.35 (1H, d, J = 1.9 Hz, H3), 7.19 (1H, dd, J = 8.4 Hz, J = 1.9 Hz, H5), 7.05 (1H, d, J = 2.2 Hz, H3′), 6.99 (1H, dd, J = 8.6 Hz, J = 2.2 Hz, H5′), 4.05 (3H, s, OCH_3); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 161.40, 157.90, 147.72, 138.04, 132.99, 126.73, 122.04, 121.48, 121.20, 120.26, 117.24, 115.07, 113.28, 57.27; Anal. Calcd. for $C_{14}H_{11}BrClNO_3$ (356.60): C 47.15, H 3.11, N 3.93, Found: C 47.26, H 3.46, N 3.93.

*5.1.3.5. N-(5-Bromo-2-hydroxyphenyl)-4-chloro-2-methoxybenzamide (**6e**).* Yield 55.8%, grey solid; m. p. 226–228 °C; IR (ATR): 3295 (ν NH), 3150 (b, ν OH), 1644 (amide I), 1608, 1592 (ν CC aromatic), 1548 (amide II), 1503, 1482, 1457 (ν CC aromatic) cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz): δ 10.63 (1H, s, OH), 10.49 (1H, s, NH), 8.55 (1H, d, J = 2.5 Hz, H6′), 8.05 (1H, d, J = 8.5 Hz, H6), 7.37 (1H, d, J = 2.0 Hz, H3), 7.20 (1H, dd, J = 8.5 Hz, J = 2.0 Hz, H5), 7.09 (1H, dd, J = 8.6 Hz, J = 2.5 HZ, H4′), 6.86 (1H, d, J = 8.6 Hz, H3′), 4.06 (3H, s, OCH_3); ^{13}C NMR (DMSO-d_6, 125 MHz): δ 161.58, 157.94, 145.78, 138.20, 133.01, 128.67, 128.38, 126.26, 121.95, 121.54, 120.06, 116.27, 113.34, 57.31; Anal. Calcd. for $C_{14}H_{11}BrClNO_3$ (356.60): C 47.15, H 3.11, N 3.93, Found: C 47.35, H 3.40, N 3.70.

*5.1.3.6. 4-Chloro-N-(2-hydroxy-4-(trifluoromethyl)phenyl)-2-methoxybenzamide (**6f**).* Yield 51.1%, white solid; m. p. 219–221 °C; IR (ATR): 3228 (b, ν OH), 1646 (amide I), 1605, 1592 (ν CC aromatic), 1549 (amide II), 1483, 1466 (ν CC aromatic) cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz): δ 11.01 (1H, s, OH), 10.62 (1H, s, NH), 8.55 (1H, d, J = 8.3 Hz, H6′), 8.06 (1H, d, J = 8.5 Hz, H6), 7.38 (1H, d, J = 1.9 Hz, H3), 7.20 (1H, dd, J = 8.5 Hz, J = 1.9 Hz, H5), 7.19–7.12 (2H, m, H3′, H5′), 4.07 (3H, s, OCH_3); ^{13}C NMR (DMSO-d_6, 125 MHz): δ 161.79, 157.97, 146.58, 138.32, 133.08, 130.75, 123.80 (1C, q, J = 31.6 Hz, C4′), 123.05 (1C, q, J = 269.8 Hz, CF_3), 121.55, 120.01, 119.59, 116.55 (1C, q, J = 3.7 Hz, C3′), 113.37, 110.65 (1C, m, C5′), 57.34; Anal. Calcd. for $C_{15}H_{11}ClF_3NO_3$ (345.70): C 52.11, H 3.21, N 4.05, Found: C 52.17, H 3.30, N 4.25.

*5.1.3.7. 4-Chloro-N-(2-hydroxy-5-(trifluoromethyl)phenyl)-2-methoxybenzamide (**6g**).* Yield 56.5%, white solid; m. p. 238–240 °C; IR (ATR): 3153 (b, ν OH), 1646 (amide I), 1620, 1605, 1596 (ν CC aromatic), 1567 (amide II), 1513, 1484, 1463, 1449 (ν CC aromatic) cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz): δ 11.25 (1H, s, OH), 10.58 (1H, s, NH), 8.74 (1H, d, J = 2.2 Hz, H6′), 8.06 (1H, d, J = 8.5 Hz, H6), 7.37 (1H, d, J = 2.0 Hz, H3), 7.30 (1H, dd, J = 8.4 Hz, J = 2.2 Hz, H4′), 7.21 (1H, dd, J = 8.5 Hz, J = 2.0 Hz, H5), 7.06 (1H, d, J = 8.4 Hz, H3′), 4.07 (3H, s, OCH_3); ^{13}C NMR (DMSO-d_6, 125 MHz): δ 161.82, 157.97, 149.65, 138.27, 133.01, 127.53, 125.74 (1C, q, J = 269.7 Hz, CF_3), 121.57, 121.18 (1C, q, J = 4.0 Hz, C6′), 120.00, 119.86 (1C, q, J = 31.6 Hz, C5′), 116.22 (1C, q, J = 4.0 Hz, C4′), 114.67, 113.36, 57.34; Anal. Calcd. for $C_{15}H_{11}ClF_3NO_3$ (345.70): C 52.11, H 3.21, N 4.05, Found: C 52.31, H 3.37, N 4.04.

*5.1.3.8. 5-Chloro-N-(4-chloro-2-hydroxyphenyl)-2-methoxybenz amide (**6h**).* Yield 69.0%, grey solid; m. p. 228–230 °C; IR (ATR):

3600–2849 (b, ν OH), 1651 (amide I), 1614, 1593 (ν CC aromatic), 1558 (amide II), 1508, 1479 (ν CC aromatic) cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz): δ 10.83 (1H, bs, OH), 10.52 (1H, s, NH), 8.34 (1H, d, $J = 8.6$ Hz, H6′), 7.98 (1H, d, $J = 2.8$ Hz, H6), 7.62 (1H, dd, $J = 8.9$ Hz, $J = 2.8$ Hz, H4), 7.30 (1H, d, $J = 8.9$ Hz, H3), 6.92 (1H, d, $J = 2.3$ Hz, H3′), 6.87 (1H, dd, $J = 8.6$ Hz, $J = 2.3$ Hz, H5′), 4.03 (3H, s, OCH_3); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 160.96, 156.16, 147.69, 133.19, 130.56, 127.35, 126.21, 125.38, 122.88, 120.89, 119.10, 115.11, 114.50, 57.18; Anal. Calcd. for $C_{14}H_{11}Cl_2NO_3$ (312.15): C 53.87, H 3.55, N 4.49, Found: C 53.46, H 3.74, N 4.40.

*5.1.3.9. 5-Chloro-N-(5-chloro-2-hydroxyphenyl)-2-methoxybenza mide (**6i**) [6].* Yield 71.8%, grey solid; m. p. 232–234 °C; IR (ATR): 3296 (b, ν OH), 1648 (amide I), 1612, 1595 (ν CC aromatic), 1551 (amide II), 1498, 1483 (ν CC aromatic) cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz): δ 10.60 (1H, s, OH), 10.58 (1H, s, NH), 8.40 (1H, d, $J = 2.7$ Hz, H6′), 7.98 (1H, d, $J = 2.8$ Hz, H6), 7.62 (1H, dd, $J = 8.9$ Hz, $J = 2.8$ Hz, H4), 7.30 (1H, d, $J = 8.9$ Hz, H3), 6.98 (1H, dd, $J = 8.5$ Hz, $J = 2.7$ Hz, H4′), 6.90 (1H, d, $J = 8.5$ Hz, H3′), 4.03 (3H, s, OCH_3); ^{13}C NMR (DMSO-d_6, 125 MHz): δ 161.15, 156.19, 145.42, 133.31, 130.57, 128.13, 125.40, 123.50, 122.71, 122.66, 119.27, 115.73, 115.14, 57.19; Anal. Calcd. for $C_{14}H_{11}Cl_2NO_3$ (312.15): C 53.87, H 3.55, N 4.49, Found: C 54.31, H 3.91, N 4.31.

*5.1.3.10. 5-Chloro-N-(4,5-dichloro-2-hydroxyphenyl)-2-methoxybenzamide (**6j**).* Yield 68.4%, white solid; m. p. 248–250 °C; IR (ATR): 3294 (b, ν OH), 1649 (amide I), 1593 (ν CC aromatic), 1541 (amide II), 1498, 1479 (ν CC aromatic) cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz): δ 11.07 (1H, s, OH), 10.55 (1H, s, NH), 8.53 (1H, s, H6′), 7.95 (1H, d, $J = 2.8$ Hz, H6), 7.60 (1H, dd, $J = 8.8$ Hz, $J = 2.8$ Hz, H4), 7.27 (1H, d, $J = 8.8$ Hz, H3), 7.02 (1H, s, H3′), 4.01 (3H, s, OCH_3); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 161.14, 156.17, 146.38, 133.39, 130.55, 127.32, 125.42, 124.85, 122.32, 120.58, 120.33, 115.60, 115.04, 57.16; Anal. Calcd. for $C_{14}H_{10}Cl_3NO_3$ (346.59): C 48.52, H 2.91, N 4.04, Found: C 48.58, H 3.21, N 4.13.

*5.1.3.11. N-(4-Bromo-2-hydroxyphenyl)-5-chloro-2-methoxybenza mide (**6k**).* Yield 55.4%, white solid; m. p. 239–241 °C; IR (ATR): 3320 (ν NH), 3254 (b, ν OH), 1645 (amide I), 1613, 1595 (ν CC aromatic), 1541 (amide II), 1502, 1481, 1462 (ν CC aromatic) cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz): δ 10.80 (1H, s, OH), 10.52 (1H, s, NH), 8.29 (1H, d, $J = 8.6$ Hz, H6′), 7.98 (1H, d, $J = 2.8$ Hz, H6), 7.62 (1H, dd, $J = 8.9$ Hz, $J = 2.8$ Hz, H4), 7.30 (1H, d, $J = 8.9$ Hz, H3), 7.05 (1H, d, $J = 2.2$ Hz, H3′), 7.00 (1H, dd, $J = 8.6$ Hz, $J = 2.2$ Hz, H5′), 4.03 (3H, s, OCH_3); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 160.96, 156.15, 147.79, 133.18, 130.55, 126.59, 125.37, 122.86, 122.06, 121.28, 117.27, 115.25, 115.10, 57.17; Anal. Calcd. for $C_{14}H_{11}BrClNO_3$ (356.60): C 47.15, H 3.11, N 3.93, Found: C 46.92, H 3.31, N 4.02.

*5.1.3.12. N-(5-Bromo-2-hydroxyphenyl)-5-chloro-2-methoxybenza mide (**6l**).* Yield 64.7%, grey solid; m. p. 223–224 °C; IR (ATR): 3301 (b, ν OH), 1652 (amide I), 1610, 1595 (ν CC aromatic), 1542 (amide II), 1481, 1458 (ν CC aromatic) cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz): δ 10.64 (1H, s, OH), 10.57 (1H, s, NH), 8.52 (1H, d, $J = 2.5$ Hz, H6′), 7.97 (1H, d, $J = 2.8$ Hz, H6), 7.62 (1H, dd, $J = 8.9$ Hz, $J = 2.8$ Hz, H4), 7.29 (1H, d, $J = 8.9$ Hz, H3), 7.09 (1H, dd, $J = 8.6$ Hz, $J = 2.5$ Hz, H4′), 6.85 (1H, d, $J = 8.6$ Hz, H3′), 4.02 (3H, s, OCH_3); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 161.15, 156.20, 145.88, 133.35, 130.58, 128.54, 126.45, 125.41, 122.66, 122.04, 116.33, 115.16, 110.30, 57.21; Anal. Calcd. for $C_{14}H_{11}BrClNO_3$ (356.60): C 47.15, H 3.11, N 3.93, Found: C 47.03, H 3.43, N 4.04.

*5.1.3.13. 5-Chloro-N-(2-hydroxy-4-(trifluoromethyl)phenyl)-2-methoxybenzamide (**6m**).* Yield 60.5%, white solid; m. p. 207–209 °C; IR (ATR): 3253 (b, ν OH), 1651 (amide I), 1610, 1598 (ν CC aromatic), 1545 (amide II), 1482 (ν CC aromatic) cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz): δ 11.00 (1H, s, OH), 10.70 (1H, s, NH), 8.53 (1H, d, $J = 8.3$ Hz, H6′), 7.99 (1H, d, $J = 2.9$ Hz, H6), 7.61 (1H, dd, $J = 8.9$ Hz, $J = 2.9$ Hz, H4), 7.30 (1H, d, $J = 8.9$ Hz, H3), 7.17 (1H, dd, $J = 8.3$ Hz, $J = 2.1$ Hz, H5′), 7.15 (1H, d, $J = 2.1$ Hz, H3′), 4.04 (3H, s, OCH_3); ^{13}C NMR (DMSO-d_6, 125 MHz): δ 161.32, 156.21, 146.65, 133.39, 130.62, 125.42, 124.27 (1C, q, $J = 270.0$ Hz, CF_3), 123.95 (1C, q, $J = 31.6$ Hz, C4′), 122.58, 119.66, 116.52 (1C, q, $J = 4.0$ Hz, C3′), 115.11, 110.69 (1C, q, $J = 3.8$ Hz, C5′), 57.19; Anal. Calcd. for $C_{15}H_{11}ClF_3NO_3$ (345.70): C 52.11, H 3.21, N 4.05, Found: C 52.35, H 3.38, N 4.23.

*5.1.3.14. 5-Chloro-N-(2-hydroxy-5-(trifluoromethyl)phenyl)-2-methoxybenzamide (**6n**).* Yield 57.9%, white solid; m. p. 190–192 °C; IR (ATR): 3182 (b, ν OH), 1648 (amide I), 1620, 1605 (ν CC aromatic), 1565 (amide II), 1513, 1484, 1449 (ν CC aromatic) cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz): δ 11.26 (1H, s, OH), 10.66 (1H, s, NH), 8.72 (1H, d, $J = 2.3$ Hz, H6′), 7.99 (1H, d, $J = 2.9$ Hz, H6), 7.63 (1H, dd, $J = 8.9$ Hz, $J = 2.9$ Hz, H4), 7.31 (1H, d, $J = 8.9$ Hz, H3), 7.31 (1H, m, H4′), 7.07 (1H, d, $J = 8.4$ Hz, H3′), 4.04 (3H, s, OCH_3); ^{13}C NMR (DMSO-d_6, 125 MHz): δ 161.38, 156.21, 149.73, 133.37, 130.59, 127.39, 125.42, 125.33 (1C, q, $J = 269.4$ Hz, CF_3), 122.61, 121.35 (1C, q, $J = 4.0$ Hz, C6′), 119.78 (1C, q, $J = 31.7$ Hz, C5′), 116.31 (1C, q, $J = 4.0$ Hz, C4′), 115.15, 114.72, 57.22; Anal. Calcd. for $C_{15}H_{11}ClF_3NO_3$ (345.70): C 52.11, H 3.21, N 4.05, Found: C 52.28, H 2.83, N 4.13.

*5.1.3.15. 5-Chloro-2-(4-chloro-2-methoxybenzamido)phenyl 4-chloro-2-methoxybenzoate (**7a**).* By-product, yield 8.2%, white solid; m. p. 153–155 °C; IR (ATR): 3341 (ν NH), 1754 (ν CO, ester), 1665 (amide I), 1594, 1565 (ν CC aromatic), 1534 (amide II), 1485, 1462, 1455 (ν CC aromatic) cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): δ 9.74 (1H, s, NH), 8.48 (1H, d, $J = 9.5$ Hz, H3′), 8.17 (1H, d, $J = 8.4$ Hz), 8.01 (1H, d, $J = 8.0$ Hz), 7.31–7.23 (2H, m), 7.12–7.03 (3H, m), 6.88 (1H, d, $J = 1.8$ Hz), 3.86 (3H, s, OCH_3), 3.52 (3H, s, OCH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 162.47, 162.34, 160.66, 157.50, 141.36, 140.83, 139.15, 133.67, 133.55, 129.60, 128.89, 126.63, 123.45, 122.45, 121.92, 120.81, 120.09, 116.30, 113.03, 112.11, 56.34, 55.95; Anal. Calcd. for $C_{22}H_{16}Cl_3NO_5$ (480.73): C 54.97, H 3.35, N 2.91, Found: C 55.26, H 3.68, N 2.85.

*5.1.3.16. 2-(4-Chloro-2-methoxybenzamido)-5-(trifluoromethyl) phenyl 4-chloro-2-methoxybenzoate (**7b**).* By-product, yield 9.5%, white solid; m. p. 162–164 °C; IR (ATR): 3334 (ν NH), 1758 (ν CO, ester), 1670 (amide I), 1621, 1592, 1569 (ν CC aromatic), 1537 (amide II), 1506, 1488, 1479, 1463 (ν CC aromatic) cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): δ 9.94 (1H, s, NH), 8.75 (1H, d, $J = 8.6$ Hz, H3′), 8.18 (1H, d, $J = 8.3$ Hz), 8.05 (1H, d, $J = 8.2$ Hz), 7.60–7.51 (2H, m), 7.16–7.04 (3H, m), 6.90 (1H, d, $J = 1.8$ Hz), 3.88 (3H, s, OCH_3), 3.51 (3H, s, OCH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 162.57, 162.42, 160.75, 157.52, 141.57, 139.80, 139.46, 134.08, 133.75, 133.62, 125.94 (1C, q, $J = 33.3$ Hz, C5′), 123.68 (1C, q, $J = 3.8$ Hz, C6′), 122.32 (1C, q, $J = 270.3$ Hz, CF_3), 122.21, 122.03, 120.87, 119.95, 119.50 (1C, q, $J = 3.9$ Hz, C4′), 116.10, 113.07, 112.19, 56.38, 56.01; Anal. Calcd. for $C_{23}H_{16}Cl_2F_3NO_5$ (514.28): C 53.72, H 3.14, N 2.72, Found: C 53.81, H 3.22, N 2.83.

*5.1.3.17. 4,5-Dichloro-2-(5-chloro-2-methoxybenzamido)phenyl 5-chloro-2-methoxybenzoate (**7c**).* By-product, yield 10.7%, orange solid; m. p. 184–185 °C; IR (ATR): 3324 (ν NH), 1766 (ν CO, ester), 1668 (amide I), 1592, 1572 (ν CC aromatic), 1523 (amide II), 1488, 1462 (ν CC aromatic) cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): δ 9.88 (1H, s, NH), 8.78 (1H, s, H6′), 8.18 (1H, d, $J = 2.8$ Hz), 8.02 (1H, d, $J = 2.8$ Hz), 7.57 (1H, dd, $J = 9.0$ Hz, $J = 2.8$ Hz), 7.40 (1H, dd, $J = 8.8$ Hz, $J = 2.8$ Hz), 7.41 (1H, s, H3′), 7.02 (1H, d, $J = 9.0$ Hz), 6.85 (1H, d, $J = 8.8$ Hz), 3.86 (3H, s, OCH_3), 3.54 (3H, s, OCH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 161.97, 161.79, 158.70, 155.71, 138.80, 134.97, 133.25, 132.15, 131.86, 130.27, 129.01, 128.20, 127.13, 126.94, 125.55, 123.62, 123.34, 122.53, 113.79, 112.99,

56.40, 56.09; Anal. Calcd. for $C_{22}H_{15}Cl_4NO_5$ (515.17): C 51.29, H 2.93, N 2.72, Found: C 51.66, H 3.32, N 2.63.

*5.1.3.18. 2-(5-Chloro-2-methoxybenzamido)-5-(trifluoromethyl) phenyl 5-chloro-2-methoxybenzoate (**7d**).* By-product, yield 7.8%, white solid; m. p. 142–143 °C; IR (ATR): 3333 (ν NH), 1760 (ν CO, ester), 1681 (amide I), 1619, 1599 (ν CC aromatic), 1541 (amide II), 1489, 1482, 1463 (ν CC aromatic) cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): δ 10.02 (1H, s, NH), 8.76 (1H, d, J = 8.5 Hz, H3′), 8.21 (1H, d, J = 2.8 Hz), 8.06 (1H, d, J = 2.7 Hz), 7.59 (1H, d, J = 2.5 Hz), 7.58–7.53 (2H, m), 7.42 (1H, dd, J = 8.8 Hz, J = 2.8 Hz), 7.03 (1H, d, J = 8.9 Hz), 6.86 (1H, d, J = 8.8 Hz), 3.87 (3H, s, OCH_3), 3.55 (3H, s, OCH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 162.21, 161.96, 158.71, 155.73, 139.73, 134.91, 133.91, 133.26, 132.23, 131.91, 127.15, 125.73 (1C, q, J = 33.4 Hz, C5′), 125.55, 123.77 (1C, q, J = 3.8 Hz, C6′), 123.26 (1C, q, J = 270.1 Hz, CF_3), 122.74, 122.25, 119.50 (1C, q, J = 3.8 Hz, C4′), 118.95, 113.77, 113.00, 56.41, 56.10; Anal. Calcd. for $C_{23}H_{16}Cl_2F_3NO_5$ (514.28): C 53.72, H 3.14, N 2.72, Found: C 53.90, H 3.29, N 2.95.

*5.1.4. General procedure for the preparation of substituted 2-methoxy-2′-hydroxybenzothioanilides (**8**)*

To a solution containing 1.3 mmol of the benzanilide derivative (**6**) in 10 mL of pyridine, 2.8 mmol of P_4S_{10} was added, and the reaction mixture was stirred and refluxed for 4 h. Then, 40 mL of $CHCl_3$, 40 mL of a 5% solution of HCl and 10 mL of concentrated HCl were added, and the mixture was refluxed under vigorous stirring for 1 h. The organic phase was removed, and the aqueous phase was extracted three times with $CHCl_3$ (50 mL). Then, the organic phases were collected, dried with Na_2SO_4 and evaporated. The oil residue was purified by column chromatography and crystallisation [18].

*5.1.4.1. 4-Chloro-N-(4-chloro-2-hydroxyphenyl)-2-methoxybenzothioamide (**8a**).* Yield 35.9%, yellow solid; m. p. 168–169 °C, decomposition; IR (ATR): 3132 (bp, ν OH), 1620, 1590, 1557, 1499, 1476, 1456 (ν CC aromatic) cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz): δ 11.34 (1H, s, OH), 10.52 (1H, s, NH), 8.09 (1H, d, J = 8.5 Hz, H6′), 7.78 (1H, d, J = 8.4 Hz, H6), 7.22 (1H, d, J = 1.9 Hz, H3), 7.09 (1H, dd, J = 8.4 Hz, J = 1.9 Hz, H5), 6.96 (1H, d, J = 2.3 Hz, H3′), 6.91 (1H, dd, J = 8.5 Hz, J = 2.3 Hz, H5′), 3.90 (3H, s, OCH_3); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 193.49, 155.28, 151.71, 135.70, 132.96, 130.88, 130.05, 126.88, 126.43, 120.40, 118.51, 115.66, 112.49, 56.68; Anal. Calcd. for $C_{14}H_{11}Cl_2NO_2S$ (328.21): C 51.23, H 3.38, N 4.27, S 9.77, Found: C 51.14, H 3.36, N 4.24, S 10.02.

*5.1.4.2. 4-Chloro-N-(5-chloro-2-hydroxyphenyl)-2-methoxybenzothioamide (**8b**).* Yield 27.8%, yellow solid; m. p. decomposition; IR (ATR): 3198 (bp, ν OH), 1613, 1589, 1555, 1493, 1475, 1457 (ν CC aromatic) cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz): δ 11.45 (1H, s, OH), 10.40 (1H, s, NH), 8.32 (1H, d, J = 2.6 Hz, H6′), 7.83 (1H, d, J = 8.5 Hz, H6), 7.23 (1H, d, J = 1.9 Hz, H3), 7.17 (1H, dd, J = 8.7 Hz, J = 2.6 Hz, H4′), 7.10 (1H, dd, J = 8.5 Hz, J = 1.9 Hz, H5), 6.96 (1H, d, J = 8.7 Hz, H3′), 3.92 (3H, s, OCH_3); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 193.33, 155.32, 149.40, 135.98, 133.25, 129.78, 128.30, 127.08, 124.42, 121.61, 120.51, 117.15, 112.57, 56.76; Anal. Calcd. for $C_{14}H_{11}Cl_2NO_2S$ (328.21): C 51.23, H 3.38, N 4.27, S 9.77, Found: C 51.16, H 3.22, N 4.21, S 9.82.

*5.1.4.3. 4-Chloro-N-(4,5-dichloro-2-hydroxyphenyl)-2-methoxybenzothioamide (**8c**).* Yield 28.9%, yellow solid; m. p. 176–177 °C, decomposition; IR (ATR): 3130 (bp, ν OH), 1589, 1558, 1541, 1491, 1477, 1458 (ν CC aromatic) cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz): δ 11.48 (1H, s, OH), 10.88 (1H, s, NH), 8.45 (1H, s, H6′), 7.81 (1H, d, J = 8.4 Hz, H6), 7.22 (1H, d, J = 2.0 Hz, H3), 7.12 (1H, s, H3′), 7.09 (1H, dd, J = 8.4 Hz, J = 2.0 Hz, H5), 3.90 (3H, s, OCH_3); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 193.86, 155.37, 150.49, 136.12, 133.26, 129.62, 128.62, 127.55, 126.16, 120.54, 119.72, 117.03, 112.59, 56.78; Anal. Calcd. for $C_{14}H_{10}Cl_3NO_2S$ (362.66): C 46.37, H 2.78, N 3.86, S 8.84, Found: C 46.20, H 2.58, N 3.83, S 8.86.

*5.1.4.4. N-(4-Bromo-2-hydroxyphenyl)-4-chloro-2-methoxybenzothioamide (**8d**).* Yield 25.2%, yellow solid; m. p. 167–169 °C, decomposition; IR (ATR): 3197 (bp, ν OH), 1613, 1591, 1557, 1497, 1476, 1461, 1454 (ν CC aromatic) cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz): δ 11.34 (1H, s, OH), 10.52 (1H, s, NH), 8.05 (1H, d, J = 8.6 Hz, H6′), 7.78 (1H, d, J = 8.3 Hz, H6), 7.22 (1H, d, J = 1.9 Hz, H3), 7.13–7.06 (2H, m, H5, H3′), 7.04 (1H, dd, J = 8.6 Hz, J = 2.2 Hz, H5′), 3.90 (3H, s, OCH_3); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 193.45, 155.31, 151.85, 135.75, 133.02, 130.07, 127.18, 126.85, 121.46, 120.43, 119.08, 118.55, 112.53, 56.71; Anal. Calcd. for $C_{14}H_{11}BrClNO_2S$ (372.66): C 45.12, H 2.98, N 3.76, S 8.60, Found: C 45.42, H 3.24, N 3.88, S 8.72.

*5.1.4.5. N-(5-Bromo-2-hydroxyphenyl)-4-chloro-2-methoxybenzothioamide (**8e**).* Yield 28.2%, yellow solid; m. p. 155–158 °C, decomposition; IR (ATR): 3189 (bp, ν OH), 1614, 1587, 1556, 1489, 1475, 1455 (ν CC aromatic) cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz): δ 11.43 (1H, s, OH), 10.41 (1H, s, NH), 8.39 (1H, d, J = 2.6 Hz, H6′), 7.81 (1H, d, J = 8.4 Hz, H6), 7.28 (1H, dd, J = 8.6 Hz, J = 2.6 Hz, H4′), 7.23 (1H, d, J = 1.8 Hz, H3), 7.10 (1H, dd, J = 8.4 Hz, J = 1.8 Hz, H5), 6.91 (1H, d, J = 8.6 Hz, H3′), 3.91 (3H, s, OCH_3); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 193.42, 155.32, 149.93, 135.94, 133.19, 130.00, 129.83, 128.71, 127.33, 120.50, 117.73, 112.57, 108.95, 56.75; Anal. Calcd. for $C_{14}H_{11}BrClNO_2S$ (372.66): C 45.12, H 2.98, N 3.76, S 8.60, Found: C 45.38, H 2.90, N 3.69, S 8.79.

*5.1.4.6. 4-Chloro-N-(2-hydroxy-4-(trifluoromethyl)phenyl)-2-methoxybenzothioamide (**8f**).* Yield 36.0%, yellow solid; m. p. 132–134 °C, decomposition; IR (ATR): 3246 (bp, ν OH), 1618, 1592, 1559, 1477, 1462 (ν CC aromatic) cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz): δ 11.51 (1H, s, OH), 10.79 (1H, bs, NH), 8.38 (1H, d, J = 8.8 Hz, H6′), 7.82 (1H, d, J = 8.5 Hz, H6), 7.24 (1H, d, J = 2.0 Hz, H3), 7.23–7.18 (2H, m, H3′, H5′), 7.11 (1H, dd, J = 8.5 Hz, J = 2.0 Hz, H5), 3.92 (3H, s, OCH_3); ^{13}C NMR (DMSO-d_6, 125 MHz): δ 194.03, 155.32, 150.87, 136.00, 133.19, 130.83, 129.91, 127.32 (1C, q, J = 31.5 Hz, C4′), 125.98, 124.11 (1C, q, J = 270.5 Hz, CF_3), 120.51, 115.47 (1C, q, J = 3.8 Hz, C3′), 112.58, 112.18 (1C, q, J = 3.8 Hz, C5′), 56.76; Anal. Calcd. for $C_{15}H_{11}ClF_3NO_2S$ (361.77): C 49.80, H 3.06, N 3.87, S 8.86, Found: C 49.95, H 3.15, N 3.99, S 8.75.

*5.1.4.7. 4-Chloro-N-(2-hydroxy-5-(trifluoromethyl)phenyl)-2-methoxybenzothioamide (**8g**).* Yield 22.2%, yellow solid; m. p. 153–155 °C, decomposition; IR (ATR): 3174 (bp, ν OH), 1626, 1591, 1573, 1557, 1476, 1461, 1449 (ν CC aromatic) cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz): δ 11.50 (1H, s, OH), 11.04 (1H, s, NH), 8.55 (1H, d, J = 2.3 Hz, H6′), 7.82 (1H, d, J = 8.4 Hz, H6), 7.49 (1H, dd, J = 8.5 Hz, J = 2.3 Hz, H4′), 7.24 (1H, d, J = 2.0 Hz, H3), 7.12 (1H, d, J = 8.5 Hz, H3′), 7.11 (1H, dd, J = 8.4 Hz, J = 2.0 Hz, H5), 3.92 (3H, s, OCH_3); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 193.92, 155.36, 153.92, 135.98, 133.16, 129.86, 127.48, 124.76 (1C, q, J = 4.2 Hz, C6′), 124.70 (1C, q, J = 269.3 Hz, CF_3), 122.41 (1C, q, J = 4.2 Hz, C4′), 120.51, 119.00 (1C, q, J = 32.2 Hz, C5′), 116.39, 112.58, 56.77; Anal. Calcd. for $C_{15}H_{11}ClF_3NO_2S$ (361.77): C 49.80, H 3.06, N 3.87, S 8.86, Found: C 49.62, H 2.93, N 3.67, S 8.64.

*5.1.4.8. 5-Chloro-N-(4-chloro-2-hydroxyphenyl)-2-methoxybenzothioamide (**8h**).* Yield 26.4%, yellow solid; m. p. 144–145 °C, decomposition; IR (ATR): 3197 (bp, ν OH), 1611, 1591, 1556, 1502, 1475, 1450 (ν CC aromatic) cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz): δ 11.42 (1H, s, OH), 10.50 (1H, s, NH), 8.03 (1H, d, J = 8.6 Hz, H6′), 7.71 (1H, d, J = 2.8 Hz, H6), 7.47 (1H, dd, J = 8.8 Hz, J = 2.8 Hz, H4), 7.16 (1H, d, J = 8.8 Hz, H3), 6.97 (1H, d, J = 2.5 Hz, H3′), 6.91 (1H, dd, J = 8.6 Hz, J = 2.5 Hz, H5′), 3.87 (3H, s, OCH_3); ^{13}C NMR (DMSO-d_6, 125 MHz): δ 193.10, 153.46, 151.88, 132.71, 131.36, 129.99, 126.48,

124.20, 119.29, 117.93, 115.28, 114.91, 113.62, 56.04; Anal. Calcd. for $C_{14}H_{11}Cl_2NO_2S$ (328.21): C 51.23, H 3.38, N 4.27, S 9.77, Found: C 50.82, H 3.13, N 4.23, S 9.81.

*5.1.4.9. 5-Chloro-N-(5-chloro-2-hydroxyphenyl)-2-methoxybenzothioamide (**8i**).* Yield 48.3%, yellow solid; m. p. 147–149 °C, decomposition; IR (ATR): 3315 (bp, ν OH), 1618, 1594, 1562, 1497, 1477, 1454 (ν CC aromatic) cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz): δ 11.52 (1H, s, OH), 10.36 (1H, s, NH), 8.23 (1H, d, $J = 2.6$ Hz, H6′), 7.76 (1H, d, $J = 2.8$ Hz, H6), 7.48 (1H, dd, $J = 8.8$ Hz, $J = 2.8$ Hz, H4), 7.18 (1H, dd, $J = 8.7$ Hz, $J = 2.6$ Hz, H4′), 7.16 (1H, d, $J = 8.8$ Hz, H3), 6.96 (1H, d, $J = 8.7$ Hz, H3′), 3.88 (3H, s, OCH_3); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 192.95, 153.49, 149.62, 132.46, 130.85, 130.67, 128.15, 127.30, 124.73, 124.22, 121.64, 117.29, 114.31, 56.66; Anal. Calcd. for $C_{14}H_{11}Cl_2NO_2S$ (328.21): C 51.23, H 3.38, N 4.27, S 9.77, Found: C 51.47, H 3.35, N 4.04, S 9.31.

*5.1.4.10. 5-Chloro-N-(4,5-dichloro-2-hydroxyphenyl)-2-methoxybenzothioamide (**8j**).* Yield 33.4%, yellow solid; m. p. decomposition; IR (ATR): 3240 (bp, ν OH), 1611, 1591, 1555, 1489, 1477, 1456 (ν CC aromatic) cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz): δ 11.55 (1H, bs, OH), 10.85 (1H, bs, NH), 8.39 (1H, s, H6′), 7.75 (1H, d, $J = 2.8$ Hz, H6), 7.48 (1H, dd, $J = 8.9$ Hz, $J = 2.8$ Hz, H4), 7.17 (1H, d, $J = 8.9$ Hz, H3), 7.13 (1H, s, H3′), 3.88 (3H, s, OCH_3); ^{13}C NMR (DMSO-d_6, 125 MHz): δ 193.43, 153.51, 150.64, 132.26, 130.94, 130.66, 128.81, 127.39, 126.39, 124.24, 119.74, 117.13, 114.31, 56.66; Anal. Calcd. for $C_{14}H_{10}Cl_3NO_2S$ (362.66): C 46.37, H 2.78, N 3.86, S 8.84, Found: C 46.25, H 2.99, N 3.96, S 8.92.

*5.1.4.11. N-(4-Bromo-2-hydroxyphenyl)-5-chloro-2-methoxybenzothioamide (**8k**).* Yield 38.9%, yellow solid; m. p. 148–149 °C, decomposition; IR (ATR): 3233 (bp, ν OH), 1613, 1594, 1556, 1497, 1476, 1451 (ν CC aromatic) cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz): δ 11.42 (1H, s, OH), 10.49 (1H, s, NH), 7.99 (1H, d, $J = 8.5$ Hz, H6′), 7.72 (1H, d, $J = 2.8$ Hz, H6), 7.47 (1H, dd, $J = 8.9$ Hz, $J = 2.8$ Hz, H4), 7.16 (1H, d, $J = 8.9$ Hz, H3), 7.11 (1H, d, $J = 2.2$ Hz, H3′), 7.04 (1H, dd, $J = 8.5$ Hz, $J = 2.2$ Hz, H5′), 3.87 (3H, s, OCH_3); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 193.01, 153.45, 151.99, 132.70, 130.68, 130.51, 127.37, 126.71, 124.15, 121.50, 119.28, 118.65, 114.26, 56.62; Anal. Calcd. for $C_{14}H_{11}BrClNO_2S$ (372.66): C 45.12, H 2.98, N 3.76, S 8.60, Found: C 45.34, H 3.20, N 3.97, S 8.76.

*5.1.4.12. N-(5-Bromo-2-hydroxyphenyl)-5-chloro-2-methoxybenzothioamide (**8l**).* Yield 38.7%, yellow solid; m. p. 148–149 °C, decomposition; IR (ATR): 3299 (bp, ν OH), 1616, 1593, 1562, 1493, 1477, 1453 (ν CC aromatic) cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz): δ 11.51 (1H, s, OH), 10.38 (1H, s, NH), 8.32 (1H, d, $J = 2.5$ Hz, H6′), 7.75 (1H, d, $J = 2.8$ Hz, H6), 7.48 (1H, dd, $J = 8.8$ Hz, $J = 2.8$ Hz, H4), 7.29 (1H, dd, $J = 8.6$ Hz, $J = 2.5$ Hz, H4′), 7.16 (1H, d, $J = 8.8$ Hz, H3), 6.92 (1H, d, $J = 8.6$ Hz, H3′), 3.88 (3H, s, OCH_3); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 193.03, 153.48, 150.13, 132.50, 130.83, 130.62, 130.20, 128.56, 127.61, 124.21, 117.85, 114.30, 108.97, 56.65; Anal. Calcd. for $C_{14}H_{11}BrClNO_2S$ (372.66): C 45.12, H 2.98, N 3.76, S 8.60, Found: C 45.12, H 2.89, N 3.77, S 8.54.

*5.1.4.13. 5-Chloro-N-(2-hydroxy-4-(trifluoromethyl)phenyl)-2-methoxybenzothioamide (**8m**).* Yield 49.0%, yellow solid; m. p. 127–129 °C, decomposition; IR (ATR): 3204 (bp, ν OH), 1622, 1595, 1568, 1476, 1458 (ν CC aromatic) cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz): δ 11.58 (1H, bs, OH), 10.77 (1H, bs, NH), 8.31 (1H, d, $J = 8.9$ Hz, H6′), 7.75 (1H, d, $J = 2.7$ Hz, H6), 7.49 (1H, dd, $J = 8.9$ Hz, $J = 2.7$ Hz, H4), 7.25–7.19 (2H, m, H3′, H5′), 7.17 (1H, d, $J = 8.9$ Hz, H3), 3.89 (3H, s, OCH_3); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 193.61, 153.49, 151.06, 132.57, 130.90, 130.69, 130.62, 127.66 (1C, q, $J = 31.7$ Hz, C4′), 126.28, 125.37 (1C, q, $J = 270.4$ Hz, CF_3), 124.25, 115.53 (1C, q, $J = 3.9$ Hz, C3′), 114.34, 112. 34 (1C, q, $J = 3.7$ Hz, C5′), 56.68; Anal. Calcd. for $C_{15}H_{11}ClF_3NO_2S$ (361.77): C 49.80, H 3.06, N 3.87, S 8.86, Found: C 49.71, H 2.85, N 3.69, S 8.92.

*5.1.4.14. 5-Chloro-N-(2-hydroxy-5-(trifluoromethyl)phenyl)-2-methoxybenzothioamide (**8n**).* Yield 26.3%, yellow solid; m. p. 132–133 °C, decomposition; IR (ATR): 3252 (bp, ν OH), 1624, 1574, 1478, 1460, 1447 (ν CC aromatic) cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz): δ 11.57 (1H, s, OH), 11.00 (1H, s, NH), 8.48 (1H, d, $J = 2.3$ Hz, H6′), 7.75 (1H, d, $J = 2.7$ Hz, H6), 7.55–7.45 (2H, m, H4, H4′), 7.17 (1H, d, $J = 8.9$ Hz, H3), 7.12 (1H, d, $J = 8.5$ Hz, H3′), 3.89 (3H, s, OCH_3); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 193.52, 154.10, 153.50, 132.53, 130.85, 130.58, 127.32, 124.93 (1C, q, $J = 3.9$ Hz, C6′), 124.21, 124.19 (1C, q, $J = 269.4$ Hz, CF_3), 122.70 (1C, q, $J = 3.8$ Hz, C4′), 119.05 (1C, q, $J = 32.2$ Hz, C5′), 116.50, 114.30, 56.66; Anal. Calcd. for $C_{15}H_{11}ClF_3NO_2S$ (361.77): C 49.80, H 3.06, N 3.87, S 8.86, Found: C 49.66, H 3.15, N 4.02, S 9.05.

*5.1.4.15. 6-Chloro-2-(4-chloro-2-methoxyphenyl)benzo[d]oxazole (**9a**).* By-product, yield 20.7%, white solid; m. p. 146–147 °C; IR (ATR): 1614, 1592, 1566, 1550, 1487, 1471, 1458, 1446 (ν CC aromatic) cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): δ 8.01 (1H, d, $J = 8.5$ Hz, H6′), 7.97 (1H, d, $J = 1.8$ Hz), 7.82 (1H, d, $J = 8.5$ Hz), 7.45 (1H, dd, $J = 8.5$ Hz, $J = 1.8$ Hz), 7.38 (1H, d, $J = 1.8$ Hz), 7.21 (1H, dd, $J = 8.5$ Hz, $J = 1.8$ Hz), 3.95 (3H, s, OCH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 161.41, 158.97, 150.50, 140.46, 138.17, 132.45, 129.83, 125.38, 121.05, 120.96, 114.05, 113.53, 111.60, 56.81; Anal. Calcd. for $C_{14}H_9Cl_2NO_2$ (294.13): C 57.17, H 3.08, N 4.76, Found: C 56.95, H 3.26, N 4.97.

*5.1.4.16. 5,6-Dichloro-2-(4-chloro-2-methoxyphenyl)benzo[d]oxazole (**9b**).* By-product, yield 42.8%, white solid; m. p. 184–185 °C; IR (ATR): 1605, 1592, 1573, 1536, 1479, 1465 (ν CC aromatic) cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz): δ 8.03 (1H, d, $J = 8.3$ Hz, H6′), 7.86 (1H, s), 7.68 (1H, s), 7.09 (1H, dd, $J = 8.3$ Hz, $J = 1.9$ Hz, H5′), 7.07 (1H, d, $J = 1.9$ Hz, H3′), 4.01 (3H, s, OCH_3); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 162.45, 159.03, 148.84, 141.69, 139.33, 132.10, 129.01, 128.65, 121.16, 121.08, 113.82, 112.88, 112.19, 56.49; Anal. Calcd. for $C_{14}H_8Cl_3NO_2$ (328.58): C 51.18, H 2.45, N 4.26, Found: C 51.55, H 2.63, N 4.56.

*5.1.4.17. 5-Bromo-2-(4-chloro-2-methoxyphenyl)benzo[d]oxazole (**9c**).* By-product, yield 24.8%, white solid; m. p. 138–139 °C; IR (ATR): 1612, 1597, 1572, 1536, 1492, 1479, 1467, 1445 (ν CC aromatic) cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): δ 8.05 (1H, d, $J = 8.3$ Hz, H6′), 7.93 (1H, s), 7.51–7.40 (2H, s), 7.14–7.02 (2H, m′, H3′, H5′), 4.02 (3H, s, OCH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 161.85, 158.97, 149.23, 143.55, 139.00, 132.10, 128.08, 123.10, 121.09, 117.13, 114.20, 112.85, 111.67, 56.50; Anal. Calcd. for $C_{14}H_9BrClNO_2$ (338.58): C 49.66, H 2.68, N 4.14, Found: C 49.45, H 2.90, N 4.18.

*5.1.4.18. 6-Bromo-2-(4-chloro-2-methoxyphenyl)benzo[d]oxazole (**9d**).* By-product, yield 38.1%, white solid; m. p. 157–158 °C; IR (ATR): 1608, 1596, 1576, 1538, 1481, 1471, 1458 (ν CC aromatic) cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): δ 8.04 (1H, d, $J = 8.8$ Hz, H6′), 7.74 (1H, d, $J = 1.8$ Hz, H7), 7.66 (1H, d, $J = 8.5$ Hz, H4), 7.46 (1H, dd, $J = 8.5$ Hz, $J = 1.8$ Hz, H5), 7.11–7.05 (2H, m, H3′, H5′), 4.02 (3H, s, OCH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 161.16, 158.95, 150.67, 141.21, 138.90, 132.01, 127.86, 121.15, 121.10, 118.02, 114.23, 113.96, 112.84, 56.49; Anal. Calcd. for $C_{14}H_9BrClNO_2$ (338.58): C 49.66, H 2.68, N 4.14, Found: C 49.68, H 2.83, N 4.23.

*5.1.4.19. 2-(4-Chloro-2-methoxyphenyl)-6-(trifluoromethyl)benzo[d]oxazole (**9e**).* By-product, yield 27.6%, white solid; m. p. 103–105 °C; IR (ATR): 1617, 1595, 1569, 1555, 1491, 1466 (ν CC aromatic) cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz): δ 8.09 (1H, d, $J = 8.4$ Hz, H6′), 7.88 (1H, d, $J = 8.4$ Hz, H4), 7.86 (1H, d, $J = 1.6$ Hz, H7), 7.62 (1H, dd, $J = 8.4$ Hz,

J = 1.6 Hz, H5), 7.11 (1H, dd, J = 8.4 Hz, J = 2.0 Hz, H5′), 7.09 (1H, d, J = 2.0 Hz, H3′), 4.03 (3H, s, OCH_3); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 163.12, 159.16, 149.67, 144.69, 139.40, 132.25, 127.31 (1C, q, J = 32.9 Hz, C6), 124.14 (1C, q, J = 270.3 Hz, CF_3), 121.70, 121.18, 120.50, 114.03, 112.93, 108.28, 56.50; Anal. Calcd. for $C_{15}H_9ClF_3NO_2$ (327.69): C 54.98, H 2.77, N 4.27, Found: C 55.15, H 2.98, N 4.35.

*5.1.4.20. 5-Chloro-2-(5-chloro-2-methoxyphenyl)benzo[d]oxazole (**9f**).* By-product, yield 17.9%, white solid; m. p. 136–137 °C; IR (ATR): 1613, 1599, 1578, 1532, 1478, 1464, 1449, 1434 (ν CC aromatic) cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz): δ 8.10 (1H, s, H6′), 7.78 (1H, s), 7.55–7.39 (2H, m), 7.33 (1H, d, J = 8.5 Hz), 7.01 (1H, d, J = 9.1 Hz, H3′), 4.00 (3H, s, OCH_3); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 161.51, 157.09, 148.87, 143.01, 132.69, 130.78, 129.94, 125.84, 125.59, 120.22, 116.90, 113.49, 111.26, 56.55; Anal. Calcd. for $C_{14}H_9Cl_2NO_2$ (294.13): C 57.17, H 3.08, N 4.76, Found: C 57.39, H 3.26, N 5.06.

*5.1.4.21. 5,6-Dichloro-2-(5-chloro-2-methoxyphenyl)benzo[d]oxazole (**9g**).* By-product, yield 33.2%, white solid; m. p. 201–202 °C; IR (ATR): 1597, 1575, 1558, 1529, 1481, 1437 (ν CC aromatic) cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz): δ 7.86 (1H, s, H7), 7.66 (1H, s, H4), 7.54 (1H, s), 7.27 (1H, m), 6.86 (1H, m), 3.80 (3H, s, OCH_3); ^{13}C NMR (DMSO-d_6, 125 MHz): δ 161.55, 156.70, 148.45, 141.07, 132.57, 130.15, 128.54, 128.07, 125.18, 120.56, 115.86, 113.27, 111.89, 56.07; Anal. Calcd. for $C_{14}H_8Cl_3NO_2$ (328.58): C 51.18, H 2.45, N 4.26, Found: C 51.31, H 2.64, N 4.52.

*5.1.4.22. 5-Bromo-2-(5-chloro-2-methoxyphenyl)benzo[d]oxazole (**9h**).* By-product, yield 21.8%, white solid; m. p. 121–122 °C; IR (ATR): 1598, 1577, 1531, 1481, 1460, 1444 (ν CC aromatic) cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz): δ 8.10 (1H, s, H6′), 7.94 (1H, s, H7), 7.53–7.37 (3H, s, H4, H5, H4′), 7.02 (1H, d, J = 9.1 Hz, H3′), 4.00 (3H, s, OCH_3); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 161.33, 157.10, 149.29, 143.48, 132.71, 130.80, 128.31, 125.84, 123.24, 117.23, 116.85, 113.49, 111.76, 56.56; Anal. Calcd. for $C_{14}H_9BrClNO_2$ (338.58): C 49.66, H 2.68, N 4.14, Found: C 49.34, H 2.82, N 4.28.

*5.1.4.23. 6-Bromo-2-(5-chloro-2-methoxyphenyl)benzo[d]oxazole (**9i**).* By-product, yield 28.5%, white solid; m. p. 113–115 °C; IR (ATR): 1608, 1598, 1578, 1534, 1482, 1455, 1435 (ν CC aromatic) cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): δ 8.09 (1H, d, J = 2.7 Hz, H6′), 7.75 (1H, d, J = 1.8 Hz, H7), 7.67 (1H, d, J = 8.5 Hz, H4), 7.47 (1H, dd, J = 8.5 Hz, J = 1.8 Hz, H5), 7.45 (1H, dd, J = 8.9 Hz, J = 2.7 Hz, H4′), 7.01 (1H, d, J = 8.9 Hz, H3′), 4.00 (3H, s, OCH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 160.66, 157.07, 150.73, 141.12, 132.62, 130.70, 127.96, 125.85, 121.27, 118.25, 116.86, 114.04, 113.48, 56.53; Anal. Calcd. for $C_{14}H_9BrClNO_2$ (338.58): C 49.66, H 2.68, N 4.14, Found: C 49.63, H 2.91, N 4.16.

*5.1.4.24. 2-(5-Chloro-2-methoxyphenyl)-6-(trifluoromethyl)benzo[d]oxazole (**9j**).* By-product, yield 13.4%, white solid; m. p. 82–84 °C; IR (ATR): 1618, 1599, 1578, 1551, 1533, 1497, 1479, 1464 (ν CC aromatic) cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz): δ 8.14 (1H, m, H6′), 7.94–7.85 (2H, m, H4, H7), 7.63 (1H, dd, J = 8.4 Hz, J = 1.6 Hz, H5), 7.48 (1H, m, H4′), 7.04 (1H, d, J = 8.9 Hz, H3′), 4.02 (3H, s, OCH_3); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 162.63, 157.29, 149.74, 144.58, 133.05, 130.94, 127.51 (1C, q, J = 32.7 Hz, C6), 125.93, 123.39 (1C, q, J = 270.7 Hz, CF_3), 121.76 (1C, q, J = 3.7 Hz, C7), 120.65, 116.66, 113.57, 108.37 (1C, q, J = 4.2 Hz, C5), 56.54; Anal. Calcd. for $C_{15}H_9ClF_3NO_2$ (327.69): C 54.98, H 2.77, N 4.27, Found: C 55.21, H 2.94, N 4.41.

*5.1.5. General procedure for the preparation of substituted 2-nitrophenols (**4**)*

An appropriate phenol (**3**) (150 mmol) was dissolved in glacial acetic acid (50 mL), and the solution was stirred and maintained at 40 °C. Then, a solution containing 11 mL of 65% HNO_3 and 30 mL of glacial acetic acid was added drop wise over 15 min. The mixture was stirred at r.t. for 45 min and poured into ice-water (400 mL). The aqueous mixture was extracted four times with $CHCl_3$ (100 mL). Next, the organic phases were collected, dried with Na_2SO_4, evaporated and purified by column chromatography (**4a**, **4c**, **4e**) or crystallisation from ethanol (**4b**). Compound **4d** was used for the next step without further purification [17].

*5.1.5.1. 4,5-Dichloro-2-nitrophenol (**4a**) [17].* Yield 38.0%, yellow solid; m. p. 66–68 °C; IR (ATR): 3275 (b, ν OH), 1614, 1564 (ν CC aromatic), 1513 (ν_{as} NO_2), 1457 (ν CC aromatic), 1344 (ν_s NO_2) cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): δ 10.47 (1H, s, OH), 8.23 (1H, s, H3), 7.33 (1H, s, H6); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 153.44, 142.26, 132.28, 125.83, 124.34, 121.48.

*5.1.5.2. 4-Bromo-2-nitrophenol (**4b**) [25].* Yield 54.0%, yellow solid; m. p. 90–92 °C; IR (ATR): 3274 (b, ν OH), 1612, 1601, 1571 (ν CC aromatic), 1530 (ν_{as} NO_2), 1470, 1450 (ν CC aromatic), 1323 (ν_s NO_2) cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): δ 10.49 (1H, s, OH), 8.25 (1H, d, J = 2.4 Hz, H3), 7.66 (1H, dd, J = 9.0 Hz, J = 2.4 Hz, H5), 7.08 (1H, d, J = 9.0 Hz, H6); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 154.09, 140.35, 127.30, 126.72, 121.72, 111.69.

*5.1.5.3. 5-Bromo-2-nitrophenol (**4c**) [26].* Yield 26.0%, yellow oil; IR (ATR): 3182 (b, ν OH), 1608, 1573 (ν CC aromatic), 1530 (ν_{as} NO_2), 1466, 1448 (ν CC aromatic), 1325 (ν_s NO_2) cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz): δ 10.62 (1H, s, OH), 7.97 (1H, d, J = 9.1 Hz, H3), 7.37 (1H, d, J = 2.1 Hz, H6), 7.13 (1H, dd, J = 9.1 Hz, J = 2.1 Hz, H4); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 155.26, 132.69, 132.34, 126.05, 123.88, 122.98.

*5.1.5.4. 2-Nitro-5-(trifluoromethyl)phenol (**4e**) [27].* Yield 26.1%, yellow oil; IR (ATR): 3273 (b, ν OH), 1635, 1594 (ν CC aromatic), 1542 (ν_{as} NO_2), 1487, 1448 (ν CC aromatic), 1335 (ν_s NO_2) cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): δ 10.57 (1H, s, OH), 8.23 (1H, d, J = 8.7 Hz, H3), 7.44 (1H, d, J = 1.9 Hz, H6), 7.23 (1H, dd, J = 8.7 Hz, J = 1.9 Hz, H4); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 154.81, 138.47 (1C, q, J = 33.4 Hz, C5), 135.22, 126.08, 121.50 (1C, q, J = 271.7 Hz, CF_3), 117.87 (1C, q, J = 3.9 Hz, C6), 116.64 (1C, q, J = 3.5 Hz, C4).

*5.1.6. General procedure for the preparation of substituted 2-aminophenols (**5**)*

An appropriate 2-nitrophenol (**4**) (40 mmol) was dissolved in 100 mL of glacial acetic acid and 30 mL of water. The reaction mixture was stirred and heated to reflux. Iron powder (800 mmol) was added in portions over 15 min. After the addition of the iron powder, the mixture was refluxed for 30 min and then poured into ice-water (400 mL). The mixture was extracted four times with ethyl acetate (200 mL). Then, the organic phases were collected, extracted ten times with a 5% solution of $NaHCO_3$ (150 mL) and once with a saturated solution of NaCl (200 mL) before being dried with Na_2SO_4 and evaporated. The residue was purified by column chromatography [17].

*5.1.6.1. 2-Amino-4,5-dichlorophenol (**5a**) [17].* Yield 66.3%, dark red solid; m. p. decomposition; IR (ATR): 3467, 3376 (ν NH_2), 3298 (b, ν OH), 1610, 1581, 1502 (ν CC aromatic) cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz): δ 9.73 (1H, bs, OH), 6.74 (1H, s, H6), 6.71 (1H, s, H3), 4.95 (2H, bs, NH_2); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 143.96, 137.98, 120.68, 115.88, 115.14, 114.09.

*5.1.6.2. 2-Amino-4-bromophenol (**5b**) [25].* Yield 41.7%, dark red solid; m. p. decomposition; IR (ATR): 3457, 3386 (ν NH_2), 3278 (b, ν OH), 1601, 1582, 1505 (ν CC aromatic) cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz): δ 9.26 (1H, bs, OH), 6.70 (1H, d, J = 2.5 Hz, H3), 6.54 (1H, d, J = 8.2 Hz, H6), 6.48 (1H, dd, J = 8.2 Hz, J = 2.5 Hz, H5), 4.79

(2H, bs, NH_2); ^{13}C NMR (DMSO-d_6, 125 MHz): δ 143.46, 139.08, 118.25, 116.17, 115.82, 110.84.

*5.1.6.3. 2-Amino-5-bromophenol (**5c**) [26].* Yield 60.5%, dark red solid; m. p. decomposition; IR (ATR): 3370, 3296 (ν NH_2), 3100–2300 (b, ν OH), 1600, 1501 (ν CC aromatic) cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz): δ 9.46 (1H, bs, OH), 6.75 (1H, d, $J = 2.5$ Hz, H6), 6.67 (1H, dd, $J = 8.3$ Hz, $J = 2.5$ Hz, H4), 6.51 (1H, d, $J = 8.3$ Hz, H3), 4.66 (2H, bs, NH_2); ^{13}C NMR (DMSO-d_6, 125 MHz): δ 145.33, 136.52, 122.04, 116.84, 115.53, 106.24.

*5.1.6.4. 2-Amino-4-(trifluoromethyl)phenol (**5d**) [28].* Yield 65.6%, orange solid; m. p. 115–118 °C; IR (ATR): 3391, 3318 (ν NH_2), 3061–2342 (b, ν OH), 1618, 1528, 1460 (ν CC aromatic) cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz): δ 9.85 (1H, bs, OH), 6.85 (1H, m, H5), 6.80–6.64 (2H, m, H3, H6), 4.92 (2H, bs, NH_2); ^{13}C NMR (DMSO-d_6, 125 MHz): δ 147.28 (1C, s, C1), 137.57 (1C, s, C2), 125.28 (1C, q, $J = 269.3$ Hz, CF_3), 120.23 (1C, q, $J = 31.0$ Hz, C4), 113.91 (1C, s, C6), 113.32 (1C, q, $J = 4.3$ Hz, C5), 110.11 (1C, q, $J = 3.7$ Hz, C3).

*5.1.6.5. 2-Amino-5-(trifluoromethyl)phenol (**5e**) [29].* Yield 50.5%, orange solid; m. p. 112–115 °C; IR (ATR): 3397, 3316 (ν NH_2), 3500–2300 (b, ν OH), 1611, 1527, 1444 (ν CC aromatic) cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz): δ 9.60 (1H, bs, OH), 6.92–6.82 (2H, m, H4, H6), 6.67 (1H, d, $J = 8.7$ Hz, H3), 5.18 (2H, bs, NH_2); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 143.63 (C1), 141.03 (C2), 125.54 (1C, q, $J = 268.3$ Hz, CF_3), 117.16 (1C, q, $J = 4.2$ Hz, C6), 115.87 (1C, q, $J = 31.4$ Hz, C5), 113.10 (C3), 110.42 (1C, q, $J = 3.7$ Hz, C4).

5.2. In vitro antimycobacterial assay

The *in vitro* antimycobacterial activity of the prepared compounds was determined against *M. tuberculosis* My 331/88 (dilution of strain 10^{-3}), *M. avium* My 330/88 (dilution of strain 10^{-5}), *M. kansasii* My 235/80 (dilution of strain 10^{-4}) and *M. kansasii 6509/96* (dilution of strain 10^{-4}). All of the strains were obtained from the Czech National Collection of Type Cultures (CNCTC) with the exception of *M. kansasii 6509/96*, which was clinically isolated. The antimycobacterial activity of the compounds was determined in a Šula's semisynthetic medium (SEVAC, Prague, Czech Republic) via the micromethod for the determination of the minimum inhibitory concentration (MIC) at 37 °C after 14 and 21 days and after 7, 14 and 21 days for *M. kansasii*. The tested compounds were added to the medium in DMSO solutions, and INH was used as a standard in a sterile water solution. The concentrations of the tested compounds were used as follows: 1000, 500, 250, 125, 62.5, 32, 16, 8, 4, 2, 1 and 0.5 μmol L^{-1}. The same concentrations within the range of 0.5–250 μmol L^{-1} were used for INH.

5.3. In vitro enzymatic assay

The mycobacterium H37Rv genomic DNA was used as a template. The Icl gene (Rv0467) of 1.28 kb was amplified using PCR. The amplified DNA was cloned into the pET-28b(+) plasmid vector Novagen (Merck KGaA, Darmstadt, Germany) using *Nde*I and *Hind*III restriction sites. The recombinant plasmid was transferred into *Escherichia coli* HB101. DNA sequencing was employed to confirm that the inserted coding sequence had no mutations. For bacterial expression, 25 mL culture volumes were inoculated with BL21(DE3) cells containing the recombinant plasmid and allowed to grow until an optical density of $OD_{595} = 0.6$ was achieved. Then, the culture was induced with 1 mmol L^{-1} isopropyl-β-D-thio-galactopyranoside solution and incubated at 30 °C for an additional 4 h. The cells were harvested by centrifugation at 6000*g* for 10 min. The resulting pellet was resuspended in BugBuster Protein Extraction Reagent Novagen (Merck KGaA, Darmstadt, Germany). The cell debris was removed by centrifugation, and the histidine-tagged protein was purified using an Äkta purifier (Amersham Biosciences, Valley Stream, NY, USA). The purity of the protein was confirmed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie staining of the gel. The protein concentration was determined by the Bradford method [30].

The isocitrate lyase activity was assayed according to the protocol reported by Dixon and Kornberg [31]. The enzyme assay was optimised in the final volume of 100 μL using 96-well plates (NUNC, Schoeller, Prague, Czech Republic) and the final concentration of tested compound in the reaction mixture 10 μmol L^{-1}. The reaction buffer contained 50 mmol L^{-1} of KH_2PO_4, 4 mmol L^{-1} of $MgCl_2 \cdot 6H_2O$, 4 mmol L^{-1} of phenylhydrazine hydrochloride, 12 mmol L^{-1} of cysteine, H_2O and KOH to pH 7.0. The isocitrate cleavage was measured by the change in the absorbance at 324 nm, which is associated with the formation of glyoxylate phenyl hydrazone. Each tested compound was dissolved in DMSO to prepare a 1 mmol L^{-1} solution, and 1 μL of this solution was added to 93.9 μL of reaction buffer. Then, it was added 0.1124 μL of the enzyme in phosphate buffer and glycerol solution with concentration 0.58 mg mL^{-1} (Bradford). Finally the reaction was started by the addition of 0.2 μmol of (+)-potassium Ds-threo-isocitrate in solution. Ethionamide and isoniazid were employed as negative controls (inhibition 0%), and 3-nitropropionic acid served as the positive control. All of the control compounds were added to the reaction mixture the same way like tested compounds and also their concentration in the reaction mixture was 10 μmol L^{-1}. The inhibitory activity of DMSO alone (1 μL) was subtracted from the activities of the evaluated compounds.

5.4. In vitro cytotoxicity assay

The measurement of the *in vitro* cytotoxicity of the most active prepared compounds was performed in the human hepatic cell line Hep G2 (passage 12) (ECACC, Salisbury, UK) using the CellTiter 96 AQueous One Solution Assay method (Promega G3580, East Port, Prague, Czech Republic). In the beginning, 10,000 cells were placed in each well of a 96-well plate (NUNC, Schoeller, Prague, Czech Republic). The incubation medium was composed of Minimum Essentials Eagle Medium (Sigma–Aldrich, Prague, Czech Republic), 1% glutamine, 10% foetal bovine serum (PAA, Biotech, Prague, Czech Republic) and 1% nonessential amino acids. The incubation period consisted of 24 h and was performed in an incubation device (Shel Lab, Cornelius, OR, USA) at 37 °C with a 5% atmosphere of CO_2. Before the experiment with the tested compounds, the cells were microscopically examined. Each of the tested compounds was dissolved in DMSO and evaluated in quadruplicate for each of the eight increasing concentrations. The samples of the tested compounds and control samples were incubated for 24 h at 37 °C. After the incubation period, the reagent from the kit was added to the wells according to the recommendation of the manufacturer, and the mixture was maintained at 37 °C for 1.5 h. Then, the absorbance at 490 nm was measured with a plate analyser Infinite M200 (Tecan Group, Männedorf, Switzerland). The results were statistically evaluated in Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA, USA), and the IC_{50} value for each tested compound was determined using GraphPad Prism 5.02 (GraphPad Software, San Diego, CA, USA).

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