The synthesis and antimycobacterial properties of 4-(substituted benzylsulfanyl)pyridine-2-carboxamides

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Dedicated to Prof. Rainer Beckert on the occasion of his 60th birthday

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Abstract

4-(Substituted benzylsulfanyl)pyridine-2-carboxamides **6** were synthesized by a three-step synthesis starting from 4-chloropyridine-2-carboxylic acid and substituted benzyl thiols, with the exception of nitroderivatives. The compounds were evaluated for their anti-TB activity against *M. tuberculosis*, non-tuberculous mycobacteria (*M. kansasii* and *M. avium*), and MDR strains of *M. tuberculosis*. The activities expressed as the minimum inhibitory concentration (MIC) fall into the range of 8-250 μmol/L. The substances exhibited similar activities against both sensitive and resistant strains.

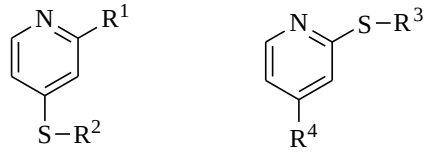
Keywords: 4-Benzylsulfanyl derivatives, pyridine-2-carboxamides, antimycobacterial activity, cytotoxicity

Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, continues to be a major threat to global public health, killing more than 1.8 million people in 2009. It has been estimated that in addition nearly 1 billion more people will be infected with TB in the next 20 years. The incidence rate of TB reached a peak worldwide in 2005; however, the total number of new cases continues to slowly rise due to population growth.[1] The control of TB is seriously threatened due

to explosive spread of the HIV epidemic, along with the increasing emergence of multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB). The increased incidence of MDR-TB and XDR-TB shows the need for further research.[2]

As a result of our anti-TB project, several papers have been published in which both synthesis and biological activity assessments have been described for a large number of 2- and 4-alkylsulfanyl derivatives of substituted pyridines (Figure 1)[3,4,5,6].



R^1= CN, $CSNH_2$, $CONHNH_2$; R^2= alkyl, substituted benzyl
R^3= substituted benzyl; R^4= CN, $CSNH_2$

Figure 1. General structures of model compounds.

For this project we have synthesized a set of 4-(substituted benzylsulfanyl)pyridine-2-carboxamides with the goal of finding further conclusions in structure-activity relationships in the group of 4-(benzylsulfanyl)-2-substituted pyridine derivatives. The prepared compounds were evaluated against *Mycobacterium tuberculosis*, non-tuberculous mycobacteria - *M. avium* and *M. kansasii*, as well as against four multi-drug resistant strains of *M. tuberculosis*.

Results and Discussion

Chemistry

The initial pyridine derivative for the synthesis of the desired sulfides **6** was selected on the basis of molecular modeling. We computed 3D models of the 4-chloropyridine-2-carboxamide and the 4-chloropyridine-2-carboxylate anion (Figure 2). The higher location of the LUMO orbital on the position 4 on pyridine ring enabled us to predict a carboxylate anion as a suitable reactant for the nucleophilic substitution. Thus, we used 4-chloropyridine-2-carboxylic acid **1** in alkaline conditions for the coupling with substituted benzyl thiols **2**.

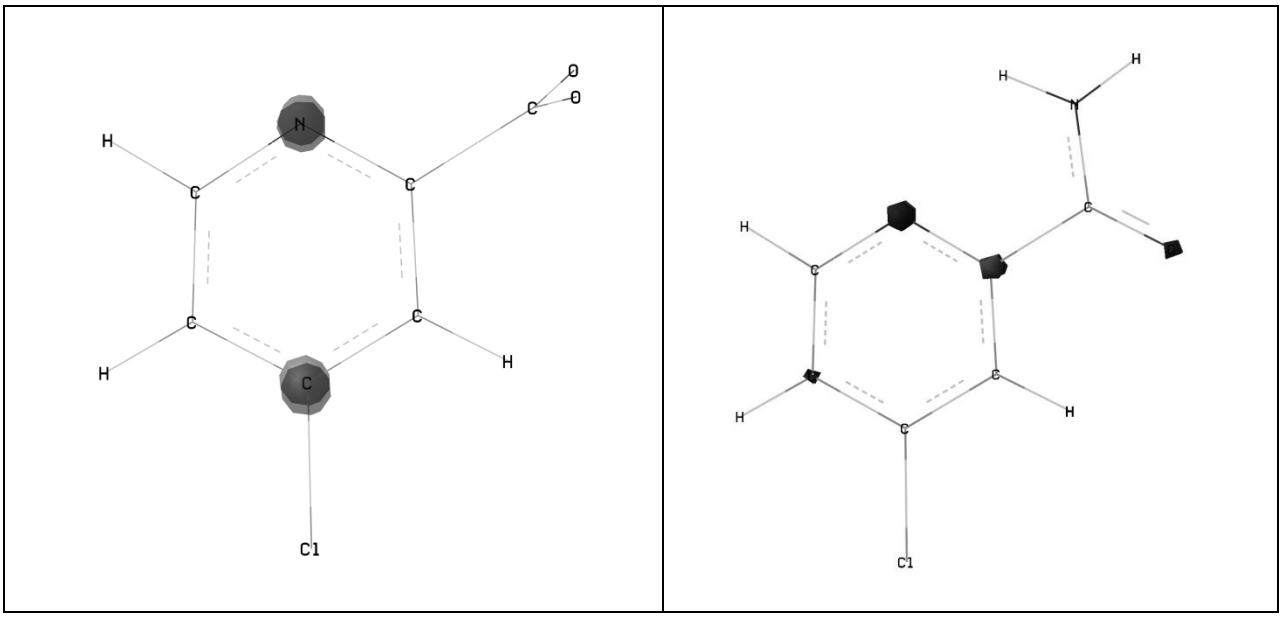
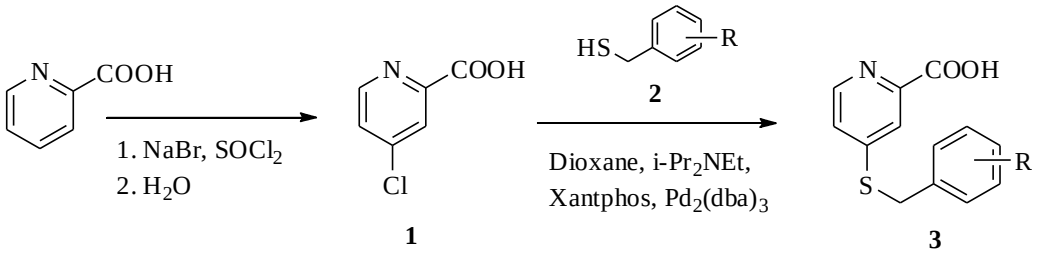


Figure 2. The dislocation of LUMO orbitals in the 4-chloropyridine-2-carboxylate anion and the 4-chloropyridine-2-carboxamide (RB3LYP/6-311+G(d,p) level, software Gaussian03W, v. 6.1; Gaussian, Inc.).

The initial 4-chloropyridine-2-carboxylic acid **1** was prepared according to the literature[7], the benzyl thiols **2** being prepared by heating appropriate benzyl halides with thiourea in ethanol, and the resulting S-alkylisothiouronium salts hydrolyzed by a sodium hydroxide solution. First, the reaction of **1** with unsubstituted benzyl thiol **2a** was studied. Nucleophilic substitution was carried out under the various conditions, the reaction conditions and yields given in Table 1. Namely, reaction in DMF and basic medium (CH_3ONa) had not given the appropriate product **3a** in good yield after 2 days. Practically the same result occurred when the reaction was carried out in DMF with K_2CO_3, using a copper catalyst. Recently, Takahiro and Toshiaki[8] reported an efficient Pd-catalyzed carbon-sulfur bond formation reaction for aryl thiols with aryl halides using $Pd_2(dba)_3$ and a phosphine ligand (Xantphos). Under these conditions, the reaction of **1** with unsubstituted benzyl thiol **2a** carried out in dioxane and i-Pr_2NEt gave the compound **3a** in good yield. In this way the set of desired compounds **3** were prepared by this reaction. The process required 9 to 12 h, depending on the thiol, and furnished products **3** in 32 to 68% yields (Scheme 1).

Table 1. Reaction conditions and yields of synthesis **3a**

Reaction conditions	Temperature / time	Yield (%)
DMF, CH_3OH, Na	r.t. / 2 days	< 5
DMF, K_2CO_3, Cu	160 °C / 6 h	15
Dioxane, i-Pr_2NEt, Xantphos, $Pd_2(dba)_3$	110 °C / 12 h	62

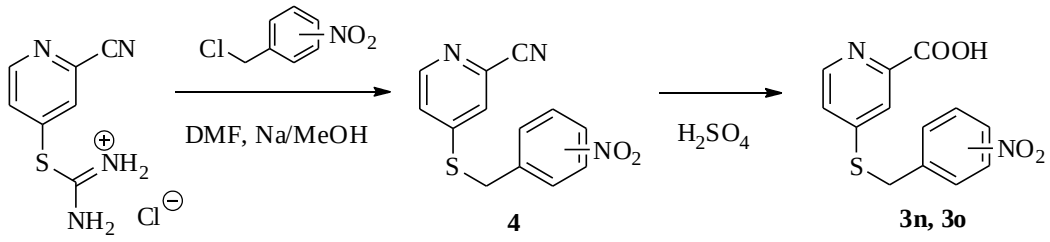


Scheme 1. Synthesis of 4-(substituted benzylsulfanyl)pyridine-2-carboxylic acids **3a-m**.

Table 2. Substituents R in compounds **2**, **3**, **5,** and **6**

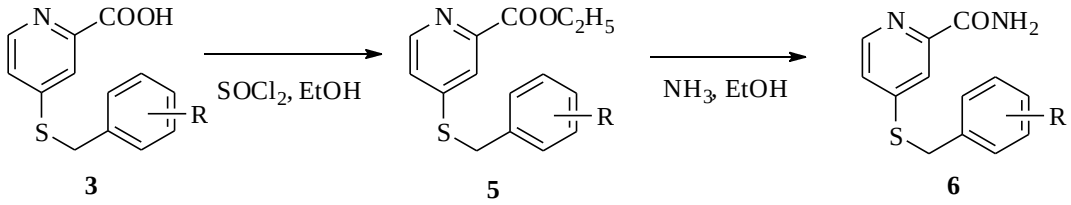
2, 3, 5, 6	R	2, 3, 5, 6	R
a	H	**i**	4-CH_3
b	3-Cl	**j**	3-CF_3
c	4-Cl	**k**	4-CF_3
d	3-F	**l**	3-CN
e	4-F	**m**	3-OCH_3
f	3-Br	**n**	4-NO_2
g	4-Br	**o**	3,5-$(NO_2)_2$
h	3-CH_3		

This procedure failed for the derivatives with the nitro group on the benzyl moiety. Nitro derivatives **3n** and **3o** were prepared by hydrolysis in 60% sulfuric acid of earlier prepared sulfide-nitriles **4**. Sulfides **4** were obtained by reacting 2-cyanopyridine-4-isothiouronium chloride with the nitrobenzyl chlorides in DMF and CH_3ONa (Scheme 2)[Error! Bookmark not defined.].



Scheme 2. Synthesis of 4-(nitrobenzylsulfanyl)pyridine-2-carboxylic acids **3n, 3o**.

The key intermediates **3** were further converted to sulfide-esters **5** by a reaction with $SOCl_2$ in ethanol. The subsequent amonolysis of **5** with ammonia gas at ambient temperature for 10-15 h afforded the desired set of compounds **6** (Scheme 3).



3 **5** **6**

Scheme 3. Synthetic route to the desired amides **6**.

Biological activity

The compounds **6** were screened for their *in vitro* antimycobacterial activity against *M. tuberculosis* and non-tuberculous mycobacteria - *M. kansasii* and *M. avium*. The minimum inhibitory concentrations (MICs) given in μmol/L are summarized in Table 3. In several cases (denoted >), the MIC could not be determined due to the limited solubility of the compounds in the test medium. As a reference, isoniazid (INH) was included in the assay.

Table 3. *In vitro* antimycobacterial activity of 4-(benzylsulfanyl)pyridine-2-carboxamides **6** expressed as MIC (μmol/L)

Compound	Strains			
6	*M. tuberculosis*	*M. kansasii*	*M. kansasii*	*M. avium*
	My 331/88	*My 235/80*	*6 509/96*	*My 330/88*
	14 d / 21 d	*7 d / 14 d / 21 d*	*7 d / 14 d / 21 d*	*14 d / 21 d*
a	>125 / 250	62 / 62/ 125	32/ 62 / 125	>62 / >62
b	62 / 125	16 / 32 / 62	32 / 62 / 62	62 / >62
c	32 / 62	32 / 32 / 32	16 / 32 / 62	32 / 32
d	125 / >250	62 / 62 / 62	62 / 125 / 125	>62 / >62
e	125 / 125	32 / 62 / 62	32 / 62 / 62	62 / 125
f	32 / 32	16 / 32 / 62	8 / 16 / 32	16 / 32
g	62 / 62	16 / 32 / 62	8 / 8 / 16	32 / 32
h	>62 / >62	16 / 32 / 32	32 / 62 / >62	62 / >62
i	62 / >62	16 / 32 / 32	32 / 62 / > 62	62 / >62
j	32 / 62	8 / 16 / 32	32 / 32 / 32	62 / 62
k	16 / 32	16 / 16 / 32	16 / 16 / 32	32 / 32
l	125 / 125	32 / >62 / >125	125 / 250 / 500	>125 / >250
m	62 / 62	16 / 62 / 62	32 / >62 / >125	>62 / >62
n	>62 / >62	>32 / >62 / >125	>16 / >32/ >62	>125 / >125
o	32 / 32	8 / 32 / 32	8 / 8 / 16	32 / 62
INH	0.5 / 1	>250 / >250 / >250	2 / 2 / 4	>250 / >250

Generally, all tested compounds showed moderate *in vitro* activity against all tested mycobacterial strains. The MIC values vary predominantly between 16 and 62 μmol/L. By

comparing their MIC values with those of INH, the compounds were less active against *M. tuberculosis* My 331/88 and *M. kansasii* 6509/96, however more active against *M. kansasii* My 235/80 and *M. avium* My 330/80. In contrast to INH, the compounds were similarly active against both *M. kansasii* strains. The advantage of the prepared compounds is their activity against *M. avium*. This strain exhibits an extreme resistance to all current antimycobacterial drugs. The obtained MIC values of compounds **6** indicate that substitution in the benzyl moiety with various electron-accepting or electron-donating substituents does not affect the antimycobacterial activity. The same conclusion was obtained for the previously synthesized 4-(substituted benzylsulfanyl)pyridine-2-carbohydrazide.[6] But, the antimycobacterial activity is strongly connected with the substituents on the pyridine moiety. In our previous paper we described the antimycobacterial activity of 4-(substituted benzylsulfanyl)pyridine-2-carbohydrazides[6], -2-carbonitriles[4], and -2-carbothioamides[4]. The newly prepared derivatives **6** have comparable activity with carbonitrile derivatives, but did not reach the activity of carbohydrazides and carbothioamides. Carbohydrazides are two to four times more active against *M. tuberculosis* My 331/88 and *M. kansasii* My 235/80 (MICs range within 4-32 μmol/L) than compounds **6** and comparable active against clinical isolate *M. kansasii* 6509/96 and *M. avium* My 330/80 (MICs range within 16-62 μmol/L). Carbothioamides (MICs ranging within 4-16 μmol/L) appear to be the most active derivatives against all tested strains.

The selected compounds **6** were further evaluated against four multidrug-resistant strains of *M. tuberculosis* isolated from TB patients. These results are summarized in Table 4.

Table 4. *In vitro* antituberculotic activity against MDR *M. tuberculosis* (MIC expressed in μmol/L)

Compound	Strains			
6	*M. tuberculosis* *7357/1998*[a] *14 d / 21 d*	*M. tuberculosis* *9449/2007*[b] *14 d / 21 d*	*M. tuberculosis* *234/2005*[c] *14 d / 21 d*	*M. tuberculosis* *8666/2010*[d] *14 d / 21 d*
b	62 / > 125	32 / > 125	125 / > 125	> 125 / > 125
d	> 125 / > 125	> 62 / > 125	> 125 / > 125	> 125 / > 125
f	> 32 / > 125	> 32 / > 125	> 32 / > 125	> 125 / > 125
h	62 / > 125	> 62 / > 125	> 125 / > 125	> 125 / > 125
j	62 / 125	62 / 125	62 / 125	> 125 / > 125
k	16 / 62	32 / 62	32 / 62	> 62 / > 62
e	> 125 / > 125	> 125 / > 125	> 125 / > 125	> 250 / > 250
o	32 / 62	> 32 / > 62	32 / 62	62 / > 62

[a]Resistant to isoniazid, rifampicin, streptomycin, ethambutol, ofloxacin, and ansamycin. [b]Resistant to isoniazid, rifampicin, streptomycin, and ansamycin. [c]Resistant to isoniazid, rifampicin, streptomycin, ethambutol, and ansamycin. [d]Resistant to isoniazid, rifampicin, streptomycin, ethambutol, ofloxacin, ansamycin, and clofazimine.

The evaluated compounds exhibit antimycobacterial activity on both drug-susceptible and drug-resistant *M. tuberculosis* strains. The activities are nearly the same against all tested strains. The comparable susceptibility of multi-drug resistant and sensitive strains of *M. tuberculosis* indicates that there is no cross resistance with current antituberculous drugs, thus the target of prepared compounds might be new.

Several compounds were assayed against cell lines HUVEC and K-562 for their antiproliferative effects (GI_{50}: concentration which inhibited cell proliferation by 50 % compared to control), and against HeLa cells for their cytotoxic effects (CC_{50}: a cytotoxic concentration which contains a specific destructive action by 50 % compared to the control; used particularly in referring to the lysis of cells). The cells were incubated with 10 concentrations of the tested compounds. The compounds **6** have low to moderate antiproliferative and cytotoxic activity (Table 5).

Table 5. Antiproliferative (GI_{50}) and cytotoxic (CC_{50}) effect (expressed in μg/mL)

Compound	Huvec (GI_{50})	K-562 (GI_{50})	HeLa (CC_{50})
6a	> 50	> 50	36.8
6c	41.9	20.6	49.7
6g	33.1	28	44.2
6i	> 50	> 50	> 50
6k	26.5	18.9	74.9

QSAR study

For the purpose of this study the MIC values of compounds **6** in μmol/L (Table 3) were expressed as log (1/MIC) = –log MIC. When the MIC value is marked with >, *i.e*. when the MIC could not be determined exactly, double the MIC value was used. The parameters taken into consideration (log *P*, Hammet constant σ, and energy of HOMO, LUMO orbitals) are summarized in Table 6.

Table 6. Physicochemial and quantum-chemical parameter values of compounds **6**

Compound	σ[9]	HOMO	LUMO	Δlog *P*
6a	0	-6.5531	-1.74154	0
6b	0.37	-6.69786	-1.84114	0.589
6c	0.22	-6.66575	-1.84522	0.583
6d	0.34	-6.68997	-1.82916	-0.027
6e	0.06	-6.64671	-1.83379	0.092
6f	0.37	-6.70194	-1.84495	0.739
6g	0.22	-6.66603	-1.84168	0.867
6h	-0.06	-6.50657	-1.7176	0.551
6i	-0.14	-6.46929	-1.7195	0.527

Table 6. (Continued)

6j	0.46	-6.79093	-1.90644	0.788
6k	0.53	-6.8127	-1.91624	0.855
6l	0.62	-6.86794	-1.9859	-0.576
6m	0.10	-6.32071	-1.69555	0.067
6n	0.81	-6.9444	-2.94049	-0.224
6o	1.42	-7.19257	-3.51465	-0.384

First, we sought relationships between MIC values and lipophilicity. In the whole set of carboxamides **6**, lipophilic parameter $\Delta\log P$ showed poor correlations with the antimycobacterial activities. The analysis through Jackknife residuals of the dependencies indicated the MIC values for two compounds, **6n** (4-NO_2) and **6o** (3,5-diNO_2) as outliers. After the outliers have been removed, statistically significant relationships were obtained. As the relationships for different incubation times are similar, only those after 14 days of incubation are presented.

$$-\log MIC\ (M.\ tuberculosis) = 0.54\ (\pm 0.16)\ \Delta\log P - 2.03\ (\pm 0.092)$$
$$n = 13,\ s = 0.243,\ r = 0.713$$
$$-\log MIC\ (M.\ kansasii) = 0.540\ (\pm 0.060)\ \Delta\log P - 1.80\ (\pm 0.035)$$
$$n = 13,\ s = 0.092,\ r = 0.938$$
$$-\log MIC\ (M.\ kansasii\ 6509/96) = 0.82\ (\pm 0.14)\ \Delta\log P - 2.00\ (\pm 0.080)$$
$$n = 13,\ s = 0.213,\ r = 0.870$$
$$-\log MIC\ (M.\ avium) = 0.64\ (\pm 0.11)\ \Delta\log P - 2.04\ (\pm 0.063)$$
$$n = 13,\ s = 0.168,\ r = 0.866$$

Antimycobacterial activities of carboxamides **6** with the exception of nitro derivatives **6n** and **6o** increased with their lipophilicity. The addition of another descriptor, *e.g*. Hammett constants or quantum chemical parameters (HOMO, LUMO energies), did not significantly improve the statistics of the relationships.

Conclusions

4-(Substituted benzylsulfanyl)pyridine-2-carboxamides **6** were synthesized by a three-step synthesis starting from 4-chloropyridine-2-carboxylic acid and substituted benzyl thiols using Pd-catalyzed cross-coupling reaction carried out in dioxane, i-Pr_2NEt, and Xantphos. The key intermediate sulfide–carboxylic acids **3** were converted to sulfide-esters **5** by a reaction with $SOCl_2$ in ethanol. The subsequent amonolysis of **5** with ammonia gas at ambient temperature afforded the desired compounds **6.** Nitroderivatives were prepared from 2-cyanopyridine-4-isothiouronium chloride and the nitrobenzyl chlorides in DMF and CH_3ONa. The prepared

sulfide-nitriles **4** afforded the key intermediates **3** by hydrolysis in 60% sulfuric acid. The compounds **6** were evaluated for their anti-TB activity against *M. tuberculosis*, non-tuberculous mycobacteria (*M. kansasii* and *M. avium*), and MDR strains of *M. tuberculosis.* The activities expressed as a minimum inhibitory concentrations fall into the range of 8-250 μmol/L. The substances exhibited similar moderate activities against both sensitive and resistant strains of *M. tuberculosis.*

Experimental Section

Chemistry

General. The melting points were determined on a Kofler block and are uncorrected. Analytical samples were dried over P_4O_{10} at 78 ºC or 25 ºC and 2.4 - 2.6 kPa for 2 hours. Elemental analyses were performed on CHNS-O CE instrument (FISONS EA 1110). IR spectra were obtained on a Nicolet Impact 400 spectrometer in KBr pellets. NMR spectra were recorded on a Varian Mercury-Vx BB 300 spectrometer operating at 300 MHz for ^{1}H and 75 MHz for ^{13}C with DMSO-d_6 as a solvent and TMS as internal standard.

Compounds **3** and **5** were obtained and characterized in our previous studies[6]; compounds **4** are described in our paper[4].

General procedures for preparation of amides 6

A two-neck, round-bottom flask equipped with a drying tube and a feeding tube was charged with a corresponding ester **5** (1.3 mmol) and 3-4 ml absolute ethanol. Dry ammonia gas was passed into the reaction solution at ambient temperature for 10-15 h. The reaction was monitored by TLC (Merck TLC plates silica gel 60 F254, aluminum back) in chloroform – methanol – triethylamine 9:1:1.25. The plates were visualized using UV light. Subsequently, the mixture was left overnight in the refrigerator. The precipitated solid was filtered off and washed with water. The crude product was purified by crystallization with ethanol.

4-(Benzylsulfanyl)pyridine-2-carboxamide (6a). White crystals, yield 42%, mp. 108-110 °C; IR (ν_{max}, cm^{-1}): 3382 (N-H), 3276 (N-H), 3200 (N-H), 1679 (C=O), 1604 (NH_2). ^{1}H-NMR (300 MHz, DMSO-*d*$_6$): δ (ppm) 8.41 (1H, dd, *J* = 5.3 Hz, *J* = 0.7 Hz, H_6), 8.10 (s, 1H, NH), 7.90 (1H, dd, *J* = 2.0 Hz, *J* = 0.7 Hz, H_3), 7.68 (1H, s, NH), 7.50 (1H, dd, *J* = 5.3 Hz, *J* = 2.0 Hz, H_5), 7.48-7.43 (2H, m, H_{Ar}), 7.38-7.24 (3H, m, H_{Ar}), 4.44 (2H, s, CH_2). ^{13}C-NMR (75 MHz, DMSO-*d*$_6$): δ (ppm) 165.8, 150.5, 150.5, 150.2, 148.3, 136.2, 129.1, 128.8, 127.6, 122.9, 118.6, 34.2; Anal. Calcd for $C_{13}H_{12}N_2OS$ (244.31) C, 63.91; H, 4.95; N, 11.47%. Found: C, 63.68; H, 4.82; N, 11.62%.

4-(3-Chlorobenzylsulfanyl)pyridine-2-carboxamide (6b). White crystals, yield 58%, mp. 131-132 °C; IR (ν_{max}, cm^{-1}): 3389 (N-H), 3165 (N-H), 1663 (C=O), 1625 (NH_2); ^{1}H-NMR (300 MHz, DMSO-*d*$_6$) δ (ppm) 8.42 (1H, dd, *J* = 5.3 Hz, *J* = 0.6 Hz, H_6), 8.09 (1H, s, NH), 7.89 (1H,

dd, *J* = 2.0 Hz, *J* = 0.6 Hz, H_3), 7.69 (1H, s, NH), 7.54 (1H, s, H_{Ar}), 7.50 (1H, dd, *J* = 5.3 Hz, *J* = 2.0 Hz, H_5), 7.44-7.31 (3H, m, H_{Ar}), 4.46 (2H, s, CH_2); ^{13}C-NMR (75 MHz, DMSO-*d*$_6$) δ (ppm) 165.8, 150.3, 150.0, 148.3, 139.0, 133.3, 130.6, 128.9, 127.7, 127.6, 123.0, 118.7, 33.5; Anal. Calcd for $C_{13}H_{11}ClN_2OS$ (278.76) C, 56.01; H, 3.98; N, 10.05%. Found: C, 56.24; H, 4.12; N, 10.23%.

4-(4-Chlorobenzylsulfanyl)pyridine-2-carboxamide (6c). Off-white crystals, yield 40%, mp. 123-124 °C; IR (ν_{max}, cm^{-1}): 3383 (N-H), 3279 (N-H), 3169 (N-H), 1682 (C=O), 1599 (NH_2); ^{1}H-NMR (300 MHz, DMSO-*d*$_6$) δ (ppm) 8.41 (1H, dd, *J* = 5.3 Hz, *J* = 0.6 Hz, H_6), 8.09 (1H, s, NH), 7.88 (1H, dd, *J* = 2.0 Hz, *J* = 0.6 Hz, H_3), 7.68 (1H, s, NH), 7.50-7.45 (3H, m, H_5, H_{Ar}), 7.42-7.37 (2H, m, H_{Ar}), 4.45 (2H, s, CH_2); ^{13}C-NMR (75 MHz, DMSO-*d*$_6$) δ (ppm) 165.8, 150.3, 150.1, 148.3, 135.5, 132.2, 130.9, 128.8, 123.0, 118.7, 33.4; Anal. Calcd for $C_{13}H_{11}ClN_2OS$ (278.76) C, 56.01; H, 3.98; N, 10.05%. Found: C, 56.30; H, 3.76; N, 10.28%.

4-(3-Fluorobenzylsulfanyl)pyridine-2-carboxamide (6d). Off-white crystals, yield 72%, mp. 144-146 °C; IR (ν_{max}, cm^{-1}) 3388 (N-H), 3278 (N-H), 3160 (N-H), 1682 (C=O), 1619 (NH_2); ^{1}H-NMR (300 MHz, DMSO-*d*$_6$) δ (ppm) 8.41 (1H, dd, *J* = 5.3 Hz, *J* = 0.6 Hz, H_6), 8.09 (1H, s, NH), 7.89 (1H, dd, *J* = 2.0 Hz, *J* = 0.6 Hz, H_3), 7.68 (1H, s, NH), 7.50 (1H, dd, *J* = 5.3 Hz, *J* = 2.0 Hz, H_5), 7.42-7.28 (3H, m, H_{Ar}), 7.14-7.06 (1H, m, H_{Ar}), 4.47 (s, 2H, CH_2); ^{13}C-NMR (75 MHz, DMSO-*d*$_6$) δ (ppm) 165.8, 162.3 (d, *J* = 244.3 Hz), 150.3, 150.1, 148.3, 139.3 (d, *J* = 7.6 Hz), 130.7 (d, *J* = 8.5 Hz), 125.2 (d, *J* = 2.9 Hz), 123.0, 118.7, 115.8 (d, *J* = 21.9 Hz), 114.5 (d, *J* = 20.9 Hz), 33.6; Anal. Calcd for $C_{13}H_{11}FN_2OS$ (262.30) C, 59.53; H, 4.23; N, 10.68%. Found: C, 59.38; H, 4.05; N, 10.44%.

4-(4-Fluorobenzylsulfanyl)pyridine-2-carboxamide (6e). Off-white crystals, yield 59%, mp. 131-135 °C; IR (ν_{max}, cm^{-1}) 3378 (N-H), 3286 (N-H), 3170 (N-H), 1687 (C=O), 1604 (NH_2); ^{1}H-NMR (300 MHz, DMSO-*d*$_6$) δ (ppm) 8.41 (1H, dd, *J* = 5.3 Hz, *J* = 0.6 Hz, H_6), 8.09 (1H, s, NH), 7.88 (1H, dd, *J* = 2.0 Hz, *J* = 0.6 Hz, H_3), 7.68 (1H, s, NH), 7.52-7.47 (3H, m, H_5, H_{Ar}), 7.21-7.13 (m, 2H, H_{Ar}), 4.44 (2H, s, CH_2); ^{13}C-NMR (75 MHz, DMSO-*d*$_6$) δ (ppm) 165.8, 161.6 (d, *J* = 243.9 Hz), 150.2, 150.3, 148.3, 132.5 (d, *J* = 3.0 Hz), 131.1 (d, *J* = 8.2 Hz), 123.0, 118.6, 115.6 (d, *J* = 21.6 Hz), 33.4; Anal. Calcd for $C_{13}H_{11}FN_2OS$ (262.30) C, 59.53; H, 4.23; N, 10.68%. Found: C, 59.31; H, 4.34; N, 10.88%.

4-(3-Bromobenzylsulfanyl)pyridine-2-carboxamide (6f). White crystals, yield 75%, mp. 123-124 °C; IR (ν_{max}, cm^{-1}) 3392 (N-H), 3285 (N-H), 3139 (N-H), 1683 (C=O), 1617 (NH_2); ^{1}H-NMR (300 MHz, DMSO-*d*$_6$) δ (ppm) 8.42 (1H, dd, *J* = 5.3 Hz, *J* = 0.6 Hz, H_6), 8.10 (1H, s, NH), 7.89 (1H, dd, *J* = 2.0 Hz, *J* = 0.6 Hz, H_3), 7.68-7.67 (2H, m, NH, H_{Ar}), 7.51-7.45 (3H, m, H_5, H_{Ar}), 7.32-7.27 (1H, m, H_{Ar}), 4.46 (2H, s, CH_2); ^{13}C-NMR (75 MHz, DMSO-*d*$_6$) δ (ppm) 165.8, 150.3, 150.0, 148.3, 139.3, 131.8, 130.9, 130.5, 128.1, 123.0, 122.0, 118.7, 33.4; Anal. Calcd for $C_{13}H_{11}BrN_2OS$ (323.21) C, 48.31; H, 3.43; N, 8.67%. Found: C, 48.58; H, 3.65; N, 8.84%.

4-(4-Bromobenzylsulfanyl)pyridine-2-carboxamide (6g). White crystals, yield 35%, mp. 141-143 °C; IR (ν_{max}, cm^{-1}) 3369 (N-H), 3151 (N-H), 1686 (C=O), 1603 (NH_2); ^{1}H-NMR (300 MHz, DMSO-*d*$_6$) δ (ppm) 8.40 (1H, dd, *J* = 5.3 Hz, *J* = 0.3 Hz, H_6), 8.08 (1H, s, NH), 7.87 (1H, dd, *J* =

2.0 Hz, *J* = 0.3 Hz, H_3), 7.67 (1H, s, NH), 7.53-7.50 (2H, m, H_{Ar}), 7.47 (1H, dd, *J* = 5.3 Hz, *J* = 2.0 Hz, H_5), 7.42-7.39 (2H, m, H_{Ar}), 4.42 (2H, s, CH_2); ^{13}C-NMR (75 MHz, DMSO-*d*$_6$) δ (ppm) 165.8, 150.3, 150.1, 148.3, 135.9, 131.7, 131.2, 123.0, 120.8, 118.7, 33.5; Anal. Calcd for $C_{13}H_{11}BrN_2OS$ (323.21) C, 48.31; H, 3.43; N, 8.67%. Found: C, 48.52; H, 3.32; N, 8.44%.

4-(3-Methylbenzylsulfanyl)pyridine-2-carboxamide (6h). Off-white crystals, yield 59%, mp. 131-132 °C; IR (ν_{max}, cm^{-1}) 3380 (N-H), 3280 (N-H), 3158 (N-H), 1687 (C=O), 1604 (NH_2); ^{1}H-NMR (300 MHz, DMSO-*d*$_6$) δ (ppm) 8.41 (1H, dd, *J* = 5.3 Hz, *J* = 0.6 Hz, H_6), 8.09 (1H, s, NH), 7.90 (1H, dd, *J* = 2.0 Hz, *J* = 0.6 Hz, H_3), 7.68 (1H, s, NH), 7.48 (1H, dd, *J* = 5.3 Hz, *J* = 2.0 Hz, H_5), 7.27-7.19 (3H, m, H_{Ar}), 7.10-7.06 (1H, m, H_{Ar}), 4.38 (2H, s, CH_2), 2.28 (3H, s, CH_3); ^{13}C-NMR (75 MHz, DMSO-*d*$_6$) δ (ppm) 165.8, 150.6, 150.2, 148.3, 138.0, 136.0, 129.7, 128.7, 128.3, 126.2, 122.8, 118.5, 34.3, 21.1; Anal. Calcd for $C_{14}H_{14}N_2OS$ (258.34) C, 65.09; H, 5.46; N, 10.84%. Found: C, 65.30; H, 5.36; N, 10.57%.

4-(4-Methylbenzylsulfanyl)pyridine-2-carboxamide (6i). Yellowish crystals, yield 40%, mp. 116-118 °C; IR (ν_{max}, cm^{-1}) 3384 (N-H), 3285 (N-H), 3193 (N-H), 1683 (C=O), 1601 (NH_2); ^{1}H-NMR (300 MHz, DMSO-*d*$_6$) δ (ppm) 8.40 (1H, dd, *J* = 5.3 Hz, *J* = 0.5 Hz, H_6), 8.09 (1H, s, NH), 7.88 (1H, dd, *J* = 2.0 Hz, *J* = 0.5 Hz, H_3), 7.68 (1H, s, NH), 7.48 (1H, dd, *J* = 5.3 Hz, *J* = 2.0 Hz, H_5), 7.38-7.28 (2H, m, H_{Ar}), 7.18-7.10 (2H, m, H_{Ar}), 4.38 (2H, s, CH_2), 2.26 (3H, s, CH_3); ^{13}C-NMR (75 MHz, DMSO-*d*$_6$) δ (ppm) 165.8, 150.6, 150.2, 148.3, 136.9, 133.0, 129.4, 129.0, 122.9, 118.6, 34.0, 20.9; Anal. Calcd for $C_{14}H_{14}N_2OS$ (258.34) C, 65.09; H, 5.46; N, 10.84%. Found: C, 65.26; H, 5.39; N, 10.98%.

4-(3-Trifluoromethylbenzylsulfanyl)pyridine-2-carboxamide (6j). Yellowish crystals, yield 58%, mp. 107-108 °C; IR (ν_{max}, cm^{-1}) 3328 (N-H), 3191 (N-H), 1684 (C=O), 1616 (NH_2), 1331 (CF_3); ^{1}H-NMR (300 MHz, DMSO-*d*$_6$) δ (ppm) 8.42 (1H, dd, *J* = 5.3 Hz, *J* = 0.6 Hz, H_6), 8.09 (1H, s, NH), 7.91 (1H, dd, *J* = 2.0 Hz, *J* = 0.6 Hz, H_3), 7.86-7.82 (1H, m, H_{Ar}), 7.80-7.74 (2H, m, H_{Ar}), 7.68 (1H, s, NH), 7.68-7.54 (2H, m, H_{Ar}), 7.51 (1H, dd, *J* = 5.3 Hz, *J* = 2.0 Hz, H_5), 4.57 (2H, s, CH_2); ^{13}C-NMR (75 MHz, DMSO-*d*$_6$) δ (ppm) 165.7, 150.3, 149.8, 148.3, 138.1, 133.2, 129.9, 129.4 (q, *J* = 31.8 Hz), 125.7 (q, *J* = 3.9 Hz), 124.4 (q, *J* = 3.9 Hz), 124.3 (q, *J* = 272.9 Hz), 123.1, 118.8, 33.5; Anal. Calcd for $C_{14}H_{11}F_3N_2OS$ (312.31) C, 53.84; H, 3.55; N, 8.97%. Found: C, 53.58; H, 3.76; N, 9.11%.

4-(4-Trifluoromethylbenzylsulfanyl)pyridin-2-carboxamide (6k). White crystals, yield 55%, mp. 123-124 °C; IR (ν_{max}, cm^{-1}) 3378 (N-H), 3290 (N-H), 3180 (N-H), 1682 (C=O), 1619 (NH_2), 1324 (CF_3); ^{1}H-NMR (300 MHz, DMSO-*d*$_6$) δ (ppm) 8.41 (1H, dd, *J* = 5.3 Hz, *J* = 0.6 Hz, H_6), 8.09 (1H, s, NH), 7.90 (1H, dd, *J* = 2.0 Hz, *J* = 0.6 Hz, H_3), 7.73-7.67 (5H, m, H_{Ar}, NH), 7.51 (1H, dd, *J* = 5.3 Hz, *J* = 2.0 Hz, H_5), 4.56 (2H, s, CH_2); ^{13}C-NMR (75 MHz, DMSO-*d*$_6$) δ (ppm) 165.7, 150.3, 149.8, 148.4, 141.5, 129.8, 128.3 (q, *J* = 31.8 Hz), 125.7 (q, *J* = 3.7 Hz), 124.4 (q, *J* = 272.9 Hz), 123.0, 118.7, 33.6; Anal. Calcd for $C_{14}H_{11}F_3N_2OS$ (312.31) C, 53.84; H, 3.55; N, 8.97%. Found: C, 53.91; H, 3.39; N, 8.78%.

4-(3-Cyanobenzylsulfanyl)pyridine-2-carboxamide (6l). White crystals, yield 60%, mp. 196-198 °C; IR (ν_{max}, cm^{-1}) 3377 (N-H), 3282 (N-H), 3161 (N-H), 2229 (CN), 1683 (C=O), 1605 (NH_2); ^{1}H-NMR (300 MHz, DMSO-*d*$_6$) δ (ppm) 8.42 (1H, dd, *J* = 5.3 Hz, *J* = 0.6 Hz, H_6), 8.09

(1H, s, NH), 7.96-7.91 (1H, m, H_{Ar}), 7.89 (1H, dd, *J* = 2.0 Hz, *J* = 0.6 Hz, H_3), 7.83-7.73 (2H, m, H_{Ar}), 7.68 (1H, s, NH), 7.61-7.51 (1H, m, H_{Ar}), 7.50 (1H, dd, *J* = 5.3 Hz, *J* = 2.0 Hz, H_5), 4.52 (2H, s, CH_2); ^{13}C-NMR (75 MHz, DMSO-*d*$_6$) δ (ppm) 165.7, 150.3, 149.7, 148.4, 138.4, 134.0, 132.6, 131.5, 130.1, 123.0, 118.7, 111.7, 33.3; Anal. Calcd for $C_{14}H_{11}N_3OS$ (269.32) C, 62.44; H, 4.12; N, 15.60%. Found: C, 62.35; H, 4.35; N, 15.81%.

4-(3-Methoxybenzylsulfanyl)pyridine-2-carboxamide (6m). White crystals, yield 30%, mp. 111-113 °C; IR (ν_{max}, cm^{-1}) 3384 (N-H), 3293 (N-H), 3220 (N-H), 1677 (C=O), 1600 (NH_2), 1050 (OCH_3); ^{1}H-NMR (300 MHz, DMSO-*d*$_6$) δ (ppm) 8.41 (1H, dd, *J* = 5.3 Hz, *J* = 0.6 Hz, H_6), 8.09 (1H, s, NH), 7.90 (1H, dd, *J* = 2.0 Hz, *J* = 0.6 Hz, H_3), 7.68 (1H, s, NH), 7.48 (1H, dd, *J* = 5.3 Hz, *J* = 2.0 Hz, H_5), 7.28-7.22 (1H, m, H_{Ar}), 7.04-7.01 (m, 2H, H_{Ar}), 6.85-6.82 (1H, m, H_{Ar}), 4.41 (2H, s, CH_2), 3.74 (s, 3H, OCH_3); ^{13}C-NMR (75 MHz, DMSO-*d*$_6$) δ (ppm) 165.8, 159.5, 150.5, 150.2, 148.3, 137.8, 129.9, 122.9, 121.2, 118.6, 114.7, 113.1, 55.2, 34.2; Anal. Calcd for $C_{14}H_{14}N_2O_2S$ (274.34) C, 61.29; H, 5.14; N, 10.21%. Found: C, 61.05; H, 5.31; N, 10.45%.

4-(4-Nitrobenzylsulfanyl)pyridine-2-carboxamide (6n). Yellow crystals, yield 60%, mp. 143-145 °C; IR (ν_{max}, cm^{-1}) 3377 (N-H), 3284 (N-H), 3185 (N-H), 1685 (C=O), 1601 (NH_2), 1519 (NO_2), 1347 (NO_2); ^{1}H-NMR (300 MHz, DMSO-*d*$_6$) δ (ppm) 8.41 (1H, dd, *J* = 5.3 Hz, *J* = 0.5 Hz, H_6), 8.27-8.13 (2H, m, H_{Ar}), 8.08 (1H, s, NH), 7.89 (1H, dd, *J* = 2.0 Hz, *J* = 0.5 Hz, H_3), 7.80-7.68 (2H, m, H_{Ar}), 7.68 (1H, s, NH), 7.51 (1H, dd, *J* = 5.3 Hz, *J* = 2.0 Hz, H_5), 4.62 (2H, s, CH_2); ^{13}C-NMR (75 MHz, DMSO-*d*$_6$) δ (ppm) 165.7, 150.4, 149.5, 148.4, 146.9, 144.8, 130.3, 123.9, 123.1, 118.8, 33.4; Anal. Calcd for $C_{13}H_{11}N_3O_3S$ (289.31) C, 53.97; H, 3.83; N, 14.52%. Found: C, 53.77; H, 3.95; N, 14.68%.

4-(3,5-Dinitrobenzylsulfanyl)pyridine-2-carboxamide (6o). Yellowish crystals, yield 35%, mp.180-182 °C; IR (ν_{max}, cm^{-1}) 3388 (N-H), 3262 (N-H), 3163 (N-H), 1676 (C=O), 1615 (NH_2), 1535 (NO_2), 1342 (NO_2); ^{1}H-NMR (300 MHz, DMSO-*d*$_6$) δ (ppm) 8.78-8.77 (2H, m, H_{Ar}), 8.71-8.70 (1H, m, H_{Ar}), 8.42 (1H, dd, *J* = 5.3 Hz, *J* = 0.5 Hz, H_6), 8.07 (1H, s, NH), 7.92 (1H, dd, *J* = 2.0 Hz, *J* = 0.5 Hz, H_3), 7.67 (1H, s, NH), 7.56 (1H, dd, *J* = 5.3 Hz, *J* = 2.0 Hz, H_5), 4.76 (2H, s, CH_2); ^{13}C-NMR (75 MHz, DMSO-*d*$_6$) δ (ppm) 165.7, 150.4, 148.9, 148.5, 148.3, 141.8, 129.4, 123.2, 119.1, 117.9, 32.6; Anal. Calcd for $C_{13}H_{10}N_4O_5S$ (334.31) C, 46.71; H, 3.02; N, 16.76%. Found: C, 46.82; H, 3.25; N, 16.59%.

Antimycobacterial evaluation

In vitro antimycobacterial activity of the compounds was evaluated against *Mycobacterium tuberculosis* CNCTC My 331/88, *Mycobacterium kansasii* CNCTC My 235/80, *Mycobacterium kansasii* 6509/96, *Mycobacterium avium* CNCTC My 330/88 and four multidrug-resistant strains of *M. tuberculosis* using the micromethod for the determination of the minimum inhibitory concentration (MIC). *M. tuberculosis* CNCTC My 331/88, *M. kansasii* CNCTC My 235/80, and *M. avium* were obtained from the Czech National Collection of Type Cultures (CNCTC); *M. kansasii* 6509/96 and multidrug-resistant strains were clinical isolates. The activities of the

compounds were determined in a Šula semisynthetic medium (SEVAC, Prague). The compounds were added to the medium in dimethylsulfoxide solutions. The following concentrations were used: 1000, 500, 250, 125, 62, 32, 16, 8, 4 and 2 μmol/L. MICs were determined after incubation at 37 °C for 14 and 21 days, and for *M. kansasii* for 7, 14, and 21 days. MIC was the lowest concentration of a substance, at which the inhibition of the growth of mycobacteria occurred. INH was used as a standard.

The resistant strains of *M. tuberculosis* may be characterized in this way: *M. tuberculosis* 735719/98 resistant to isoniazid, rifampicin, streptomycin, ethambutol, ofloxacin, and ansamycin; *M. tuberculosis* 9449/2007 resistant to isoniazid, rifampicin, streptomycin, and ansamycin; *M. tuberculosis* 234/2005 resistant to isoniazid, rifampicin, streptomycin, ethambutol, and ansamycin; *M. tuberculosis* 8666/2010 resistant to isoniazid, rifampicin, streptomycin, ethambutol, ofloxacin, ansamycin, and clofazimine.

Antiproliferative and cytotoxic assay

The target compounds were assayed against cell lines K-562 and HUVEC for their antiproliferative effects and against HeLa for their cytotoxic effects. The cells were incubated with ten concentrations of the test compounds[10].

Suspension cultures of K-562 in micro plates were analyzed by an electronic cell analyzer system CASY 1 (SCHÄRFE, Reutlingen, Germany) using an aperture of 150 μm. The software for data evaluation CASYSTAT (SCHÄRFE) offers fast graphical evaluation of the measurement parameters, e.g. as diagrams of cell diameter distributions, overlays of different curves, and cell volume distributions. The 0.2 mL content of each well in the micro plate was diluted 1:50 with CASYTON (NaCl: 7.93 g/L; Na_2EDTA: 0.38 g/L; KCl: 0.4 g/L; NaH_2PO_4 monohydrate: 0.22 g/L; NaH_2PO_4 dihydrate: 2.45 g/L; NaF: 0.3 g/L; SCHÄRFE). Every count/mL was automatically calculated from the arithmetic mean of three successive counts of 0.4 mL each. From the dose response curves the Gl50 values (concentration which inhibited cell growth by 50 %) were calculated with CASYSTAT. The Gl50 value was defined as being where the concentration-response curve intersected the 50 % line, determined by means of the cell counts/mL, compared to control.

The monolayers of the adherent HUVEC and HeLa cells were fixed by glutaraldehyde and stained with a 0.05% solution of methylene blue for 15 min. After gently washing, the stain was eluted by 0.2 mL of 0.33M HCl in the wells. The optical densities were measured at 630 nm in a DYNATECH MR 7000 micro plate reader. Comparisons of the different values were performed with Microsoft Excel.

Molecular modeling and QSAR study

Quantum-chemical calculations were performed using the program Gaussian 03W, version 6.1, revision-E.01 (Gaussian, Inc.). The geometry of the molecules was optimized using the B3LYP/6-311+G(d,p) method. Possible starting conformations for the optimizations at the DFT level were found by the conformational search module of the software HyperChem 8.0.8

(HyperCube, Inc.) on RM1 level. This method involves a random variation of dihedral angles to generate new structures and then minimizing the energy of each of these angles. Final models were visualized using the software GaussView, v. 4.1.2; (Gaussian, Inc.).

The octanol–water partition coefficients, expressed as $\log P$ values, were calculated using Advanced Chemistry Development (ACD/Labs) Software V11.02. The $\log P$ values were transformed to the increments $\Delta\log P$ by the subtraction of the value corresponding to the unsubstituted derivative.

Regression analyses were run on a PC computer using the Microsoft Excel program. In the equations, the figures in the parentheses are the standard errors of the regression coefficients, n being the number of compounds, r the correlation coefficient, and s the standard error of estimate. All equations are statistically significant at the 1% level of probability.

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Graphical Abstract

The synthesis and antimycobacterial properties of 4-(substituted benzylsulfanyl)pyridine-2-carboxamides. *Věra Klimešová, Petra Herzigová, Karel Palát, Miloš Macháček, Jiřina Stolaříková, Hans-Martin Dahse, and Ute Möllmann*