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Graphical Abstract

Synthesis and biological evaluation of 4-methyl-1,2,3-thiadiazole-5-carboxaldehyde benzoyl hydrazone derivatives

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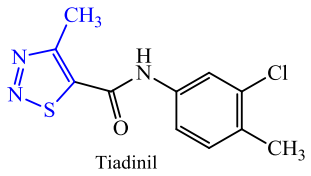
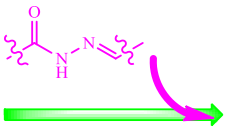
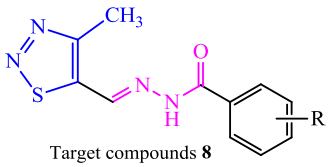


Tiadinil

Fungicidal activity against *Valsa mali*: EC_{50} = 7.65 μg/mL



Embedding carbohydrazide hydrazone bond



Target compounds **8**

Fungicidal activity against *Valsa mali:*
8g: R=4-Br , EC_{50} = 1.64 μg/mL
8n: R=4-CF_3 , EC_{50} = 1.87 μg/mL

A series of novel 4-methyl-1,2,3-thiadiazole-5-carboxaldehyde benzoyl hydrazone derivatives designed and synthesized. They were exhibited moderate to strong fungicidal activity against six fungi *in vitro* at 50 μg/mL, and some of them presented good curative activity against TMV *in vivo* at 500 μg/mL.

Original article

Synthesis and biological evaluation of 4-methyl-1,2,3-thiadiazole-5-carboxaldehyde benzoyl hydrazone derivatives

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ABSTRACT

To find new lead compounds with high biological activity, a series of novel 4-methyl-1,2,3-thiadiazole-5-carboxaldehyde benzoyl hydrazone derivatives were designed and synthesized. Their structures were confirmed by ^{1}H NMR, ^{13}C NMR, IR spectrum and elemental analysis. Preliminary bioassay indicated that the title compounds exhibited moderate to strong fungicidal activity against six fungi *in vitro* at 50 μg/mL. Moreover, some of the title compounds exhibited good curative activity against TMV *in vivo* at 500 μg/mL. The structure-activity relationship analysis of compounds against *Valsa mali* showed that compounds containing halogen at the *para* position on phenyl exhibited the best activity. Especially compound **8k** showed broad spectrum fungicidal activities against *Valsa mali*, *Botrytis cinerea*, *Pythium aphanidermatum*, *Rhizoctonia solani*, *Fusarium moniliforme* and *Alternaria solani* with the EC_{50} values of 8.20, 24.42, 15.80, 40.53, 41.48, and 34.16 μg/mL, respectively.

1. Introduction

Heterocyclic compounds, such as thiazole, pyrazole, pyridine, and triazole, play important roles in medical chemistry and agrochemicals [1]. 1,2,3-Thiadiazole, one typical heterocycle widely used in drug discovery, has increasingly attracted attention thanks to its versatile biological activities, such as anti-HIV, antitumor, antiviral, systemic acquired resistance, insecticidal, fungicidal, and herbicidal activities [2-14]. For instance, 4-methyl-1,2,3-thiadiazole derivatives **I** (Fig. 1) was an excellent necroptosis inhibitor (EC_{50}=1.0 μmol/L) [14]. Meanwhile, Fan reported a series of 4-methyl-1,2,3-thiadiazole derivatives containing 1,2,4-triazolo[3,4-b][1,3,4]thiadiazoles showed good fungicidal activity, especially compound **II** (Fig. 1) against *Rhiz octonia solani* and *Pellicularia sasakii* with EC_{50} values of 4.1 and 2.5 μg/mL, respectively [15]. Tiadinil (Fig. 1), a novel fungicide commercialized containing 1,2,3-thiadiazole, also had good induction activity against tobacco mosaic virus (TMV) [2].

Carbohydrazide hydrazone is an attractive and versatile scaffold with relevant application in several areas, including asymmetric catalysis, coordination chemistry, agrochemicals and pharmacology [16-18]. A number of carbohydrazide hydrazone derivatives possess interesting bioactivity such as antifungal, anti-inflammatory, antiplatelets, antimalarial and anticancer activities (Fig. 2) [18-22]. Metaflumizone, Diflufenzopyr, and Benquinox (Fig. 2) are known as the commercial agrochemicals containing this scaffold [16]. In view of superior properties and with the aim to find new lead compounds with high biological activity, we introduced carbohydrazide hydrazone into the structure of Tiadinil to replace the amide linkage, and designed, synthesized a series of novel 4-methyl-1,2,3-thiadiazole-5-carboxaldehyde benzoyl hydrazone derivatives (Fig. 3). All the compounds were evaluated for the activities against six plant fungal pathogens *in vitro* and tobacco mosaic virus *in vivo* (curative activity). Moreover, the initial structure-activity relationship (SAR) of these compounds was also analysized in the present work. The results might be valuable for the discovery of eco-friendly agrochemicals.

2. Results and discussion

2.1. Chemistry

The synthetic route for the title compounds **8a-8x** was illustrated in Scheme 1. The key intermediate 4-methyl-1,2,3-thiadiazole-5-carboxaldehyde **6** was synthesized from dimethyl carbonate via five steps including hydrazidation, condensation, cyclization, reduction and oxidation by conventional synthetic methods from commercially available starting materials. Then the target compounds **8a-8x**

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were easily obtained in 60–96% yields through addition-elimination condensation between compound **6** and different substituted benzoyl hydrazine **7**. Their structures were fully characterized by melting points, ^{1}H NMR, ^{13}C NMR, IR spectra and elemental analysis (Supporting information).

In order to determine the stereo structure of the target compounds, a single crystal of representative compound **8g** (R = 4-Br) was prepared by slow evaporation of a solution of compound **8g** in ethanol at 4 ℃. As shown in Fig. 4, it gives a perspective view of **8g** with the atomic labeling system, and the result demonstrates the —CH=N—NH— bond bears an (*E*) rather than (*Z*) conformation. Moreover, the crystal is distribution with dimer form and there is an intermolecular hydrogen bond between the two subunits. The crystal conformation is more stable owing to the lower energy of the system. The crystal data for **8g**: triclinic, a = 9.5767(5) Å, b = 9.6459(7) Å, c = 13.7997(9) Å, α = 84.771(6)°, β = 87.927(5)°, γ = 88.623(5)°, V = 1268.34(14) Å^3, T = 104.6, space group P-1 (no. 2), Z = 4, μ(Mo Kα) = 3.398 mm^{-1}, D_{calc} = 1.703 g/cm^3, μ=3.398 mm^{-1}, 10125 reflections measured($6.08° \leq 2\Theta \leq 52°$), 4969 unique ($R_{int}$ = 0.0263) were used in all calculations. The final R_1 was 0.0378 ($I > 2\sigma(I)$) and $wR(F_2)$ was 0.0935 (all data). Crystallographic data have been deposited with the Cambridge Crystallographic Data Centre, and the deposition number was CCDC 1481606-1481607.

2.2. In vitro antifungal activity

All the target compounds were evaluated for their fungicidal activities against six plant fungal pathogens. The results were shown in Table 1. The data suggested that all compounds had moderate to strong fungicidal activity. Compounds **8c**, **8d**, **8f**, **8g**, **8i**, **8j**, **8k**, **8m**, and **8n** exhibited more than 90% inhibition activities against *V. mali*, similar to Tiadinil (92.9%) at 50 μg/mL. Compound **8k** also displayed good activity against *B. cinerea* (87.5%), which is comparable with that of Tiadinil (80.4%) and Difenoconazole (92.1%). Unfortunately, most compounds did not show better activities for the rest plant fungal pathogens compared with Tiadinil and Difenoconazole. However, compounds **8k** and **8n** showed above 50% inhibition activity against other four fungi *P. aphanidermatum, R. solani, F. moniliforme and A. solani*. These results indicated that the title compounds are exclusively efficient against *V. mali* mycelial growth *in vitro* but not potent enough against the other five fungi. Thus, *V. mali* was chosen as the target for further study.

In order to intensively study the structure and activity relationship of the compounds against *V. mali*, therefore, different concentrations of compounds **8a-8x** were treated against *V. mali*. The EC_{50} values were calculated using linear-regression analysis, and the results were shown in Table 1. Among these 24 compounds, **8d**, **8f**, **8g**, **8i**, **8j**, and **8n** showed lower EC_{50} values than the commercial fungicide Tiadinil, especially **8g** and **8n** with the EC_{50} values of 1.6 and 1.9μg/mL, respectively, which were 4-fold higher potency than Tiadinil. The results proved that introducing carbohydrazide hydrazone into the structure of Tiadinil to replace the amide linkage is favorable to improve the fungicidal activity. However, the compounds showed weaker effectiveness than Difenoconazole.

Structure-activity relationship analysis generally indicates that: (i) The position of the substituent R on the benzene ring played an important role in fungicidal activities: *meta*-position exhibited weaker activity than the *para*-position, however, better than the *ortho*-position. Taking chloro substituents (**8h-8j**) as an instance, it is obvious that **8j** (R=4-Cl, 2.8) < **8i** (R=3-Cl, 3.8) < **8h** (R=2-Cl, 19.4) ("<" means inferior EC_{50} values). The 2, 5-dichloro substituent **8l** (36.9 μg/mL) showed the poorest activity, which is even weaker than **8h**. The 2, 4-dichloro substituent **8k** (8.2μg/mL) showed the moderate activity, which is much higher than **8h** and **8l**, but lower than **8j**. The same rule can also be found in **8b−8d** (−F), **8e−8g** (−Br), **8m−8n** (−CF_3), **8o−8q** (−NO_2) and **8r−8s** (−CH_3) groups. (ii) The electron-withdrawing groups are more favorable to the activity than the electron-donating groups in the same position. For example, when substituent R is in the *para* position, compounds with the electron-withdrawing (**8d**, **8g**, **8j**, **8n**, and **8q**,) are more potent than that with electron-donating substituent **(8s-8u)**. Among of them, except **8q** (4−NO_2, 10.2 μg/mL) is close with **8s** (4−CH_3, 10.9 μg/mL) and **8t** (4−*t*Bu, 9.4 μg/mL), and the other four compounds **8d** (4−F, 7.1 μg/mL), **8g** (4−Br, 1.6 μg/mL), **8j** (4−Cl, 2.8 μg/mL) and **8n** (4−CF_3, 1.9 μg/mL) are much higher than that of **8s** and **8t**. In conclusion, the compound containing halogen at para position exhibits the best activity.

Different from most compounds with a very narrow spectrum against the fungi tested in Table 1, compound **8k** showed broad spectrum and thus was selected to determine the EC_{50} values against the six fungi. As illustrated in Table 2, **8k** displayed different levels activity against *V. mali, B. cinerea, P. aphanidermatum, R. solani, F. moniliforme* and *A. solani* with EC_{50} values of 8.20, 24.42, 15.80, 40.53, 41.48 and 34.16 μg/mL, respectively, which are still weaker than positive control except *P. aphanidermatum*.

2.3. In vivo antivirus activity

Curative activity of all title compounds against TMV *in vivo* was evaluated. The results were shown in Table 3, in which all the compounds were active against TMV to some extent at the tested concentration. Among them, compounds **8a**, **8b**, **8e**, **8j**, **8m**, **8o**, and **8u** presented moderate curative activity at 500 μg/mL, which were higher than that of standard Tiadinil. Especially, compounds **8a** and **8b** showed good anti-TMV activity with inhibition activity of 47.2% and 49.9%, respectively, which were close to the positive control Ningnanmycin (57.9%). Interestingly, compounds containing R groups at *ortho*-position of phenyl have higher activity than those R groups at *meta*-position. The results provide useful clues for further discovery of novel lead structures possessing good antivirus activity.

3. Conclusion

A series of novel 4-methyl-1,2,3-thiadiazole-5-carboxaldehyde benzoyl hydrazone derivatives were designed and synthesized through six steps with dimethyl carbonate as starting material. The bioassay indicated that the title compounds exhibited moderate to strong antifungal activities against six fungi *in vitro* at 50 μg/mL and presented a certain degree of curative activity against TMV at

500 μg/mL. Compounds **8g** and **8n** exhibited outstanding activities against *V. mali* with the EC_{50} values of 1.64 and 1.87 μg/mL, respectively, which are 4-fold higher potency than the control Tiadinil. Moreover, compound **8k** showed broad spectrum activities against *V. mali, B. cinerea, P. aphanidermatum, R. solani, F. moniliforme and A. solani* with EC_{50} values of 8.20, 24.42, 15.80, 40.53, 41.48, and 34.16 μg/mL, respectively. This result indicated that replacing the amide linkage of Tiadinil with carbohydrazide hydrazone is favorable to improve the biological activity of lead compound. The title structure can be a new lead for further modification.

4. Experimental

4.1. Synthesis

Melting points of all compounds were determined on an X-4 binocular microscope (Fukai Instrument Co., Beijing, China), with an uncorrected thermometer. ^{1}H NMR spectra and ^{13}C NMR spectra were recorded on Bruker AM-300 (300 MHz, 75 MHz) spectrometer with $CDCl_3$ as the solvent and TMS as the internal standard. IR spectra were recorded on a Perkin Elmer Spectrum 47 100 FT-IR spectrometer with a KBr disk. Elemental analysis data were carried out on a Vario EL III elemental analyzer. Single-crystal X-ray diffraction was measured on a Gemini E single-crystal diffractometer. All the reagents were obtained commercially and used after further purification. Column chromatography purification was carried out by using silica gel (Merck 60, 200–300 mesh).

Synthesis of intermediates **2**–**6** were according the references [5, 11], the general procedures are given in the Supporting information. General procedures for target compounds **8a**–**8x**: To a solution of corresponding benzoyl hydrazine **7** (3 mmol) in anhydrous ethanol (20 mL), an intermediate **6** (0.39 g, 3 mmol) was added. The resulting mixture was then stirred at refluxed temperature, when TLC analyses indicated the disappearance of the starting material, then cooled to room temperature, followed by filtered to obtain the solid. The solid was recrystallized in ethanol to give corresponding target compounds **8**.

4.2. Bioassays

Antifungal activity (*in vitro*): The fungicidal activity was evaluated according to the mycelium growth rate method in the references at the concentration of 50μg/mL [23, 24]. Fungi used in this study included six plant fungal pathogens (*Valsa mali, Botrytis cinerea, Pythium aphanidermatum, Rhizoctonia solani, Fusarium moniliforme and Alternaria solani*). Tiadinil and Difenoconazole were used as standard and positive controls, respectively. The detailed procedures were listed in the Supporting information.

Antivirus activity (*in vivo*): The leaves of *N.tabacum* L. growing at the same ages were selected. TMV at a concentration of 0.06 μg•mL^{-1} was dipped and inoculated on the whole leaves. After the leaves were dried in the greenhouse, the compound solution was smeared on the left side, and the solvent was smeared on the right side for control. The local lesion numbers were then recorded 3–4 d after inoculation. For each compound, three repetitions were conducted. All compounds were tested at 500 μg•mL^{-1}. Tiadinil and ningnanmycin were used as standard and positive controls, respectively [25].

The activity data of curative effects against TMV was calculated by the following equation:

$$Y = \frac{CK - A}{CK} \times 100\%$$

Where: *Y* is the antivirus inhibition ratio (%), *CK* is the number of viral inflammations on the control leaves *in vivo*, and *A* is the number of viral inflammations on the target compound treated leaves in *vivo*.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at:

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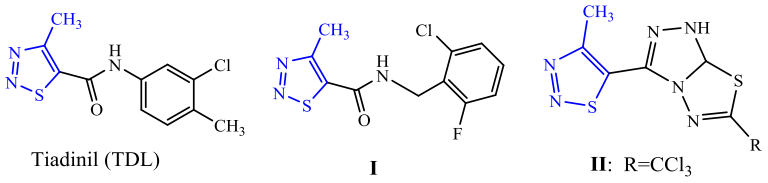


Fig. 1 The chemical structures of compounds containing 1,2,3-thiadiazole.

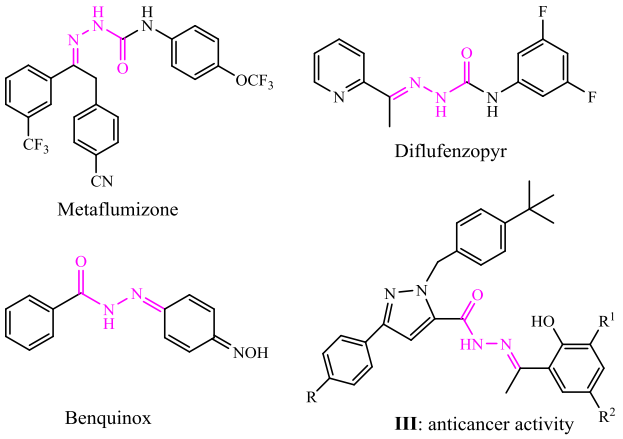


Fig. 2 The chemical structures of compounds containing carbohydrazide hydrazone

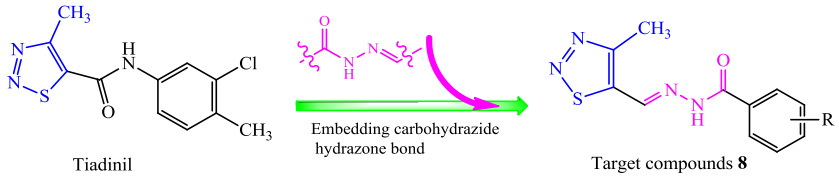


Fig. 3 Design strategy of the target compounds

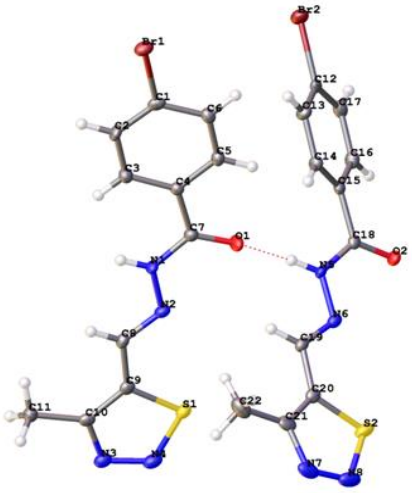


Fig. 4 The X-ray crystal structure of **8g**

Table 1 The *in vitro* antifungal activities of title compounds **8a-8x**

Compd.	Inhibitory rate of compounds at concentration 50 μg/mL (%)						*V. mali*
	V. mali	*B. cinerea*	*P. aphanidermatum*	*R. solani*	*F. moniliforme*	*A. solani*	EC_{50} (μg/mL)
8a	72.3	14.0	7.9	14.8	17.4	13.8	15.0
8b	36.6	4.2	3.2	14.5	17.8	11.7	>50
8c	97.3	63.8	54.1	27.7	22.5	28.3	9.4
8d	91.0	54.0	48.4	34.7	35.3	31.7	7.1
8e	55.9	41.1	45.1	12.2	19.8	0.0	40.9
8f	98.9	12.5	8.9	8.2	15.1	29.2	5.0
8g	99.2	60.8	40.4	36.9	38.8	36.4	1.6
8h	71.0	20.0	35.0	7.8	19.0	19.8	19.4
8i	92.2	52.5	28.0	12.6	14.3	28.8	3.8
8j	100.0	74.7	42.4	39.2	31.0	45.8	2.8
8k	91.7	87.5	81.3	60.2	71.7	58.8	8.2
8l	52.2	12.1	4.6	10.8	7.4	6.6	36.9
8m	93.8	77.0	48.1	44.3	38.8	52.2	7.9
8n	100.0	70.9	51.8	68.3	65.9	49.7	1.9
8o	16.3	3.8	4.9	4.1	17.1	10.8	>50
8p	56.4	13.6	41.1	11.5	7.4	23.2	46.8
8q	82.1	19.6	28.0	9.7	15.9	26.2	10.2
8r	50.7	15.1	28.0	24.0	19.4	21.9	>50
8s	75.4	19.2	5.6	10.0	24.4	13.4	10.9
8t	72.4	32.1	17.6	21.1	32.2	30.0	9.4
8u	58.9	14.3	18.3	15.6	16.3	23.2	25.2
8v	56.7	20.8	34.7	19.6	3.5	11.3	20.8
8w	72.8	3.8	4.9	16.7	22.5	27.0	10.9
8x	16.5	6.4	2.6	3.4	13.2	0.0	>50
Tiadinil	92.9	80.4	51.1	68.3	56.9	55.6	7.6
Difenoconazole	100.0	92.1	55.8	82.7	93.0	77.0	0.02

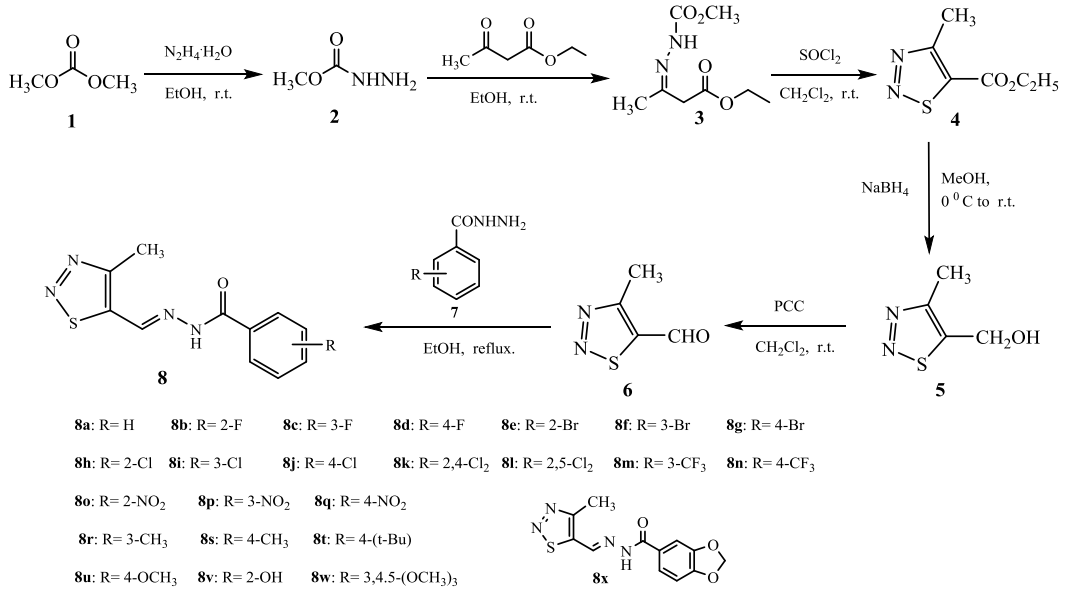
Table 2 The EC_{50} values of compound **8k** against six fungal pathogens

Compd.	EC_{50} (μg/mL)					
	V. mali	*B. cinerea*	*P. aphanidermatum*	*R. solani*	*F. moniliforme*	*A. solani*
8k	8.2	24.4	15.8	40.5	41.5	34.2
Tiadinil	7.7	6.7	39.4	10.5	NT[a]	NT
Difenoconazole	0.02	0.5	31.5	0.7	0.9	0.6

[a] NT = not tested

Table 3 The curative effects of test compounds against TMV *in vivo* (500 μg/mL).

Compd.	Curative effect (%)	Compd.	Curative effect (%)	Compd.	Curative effect (%)
8a	44.3±4.4	**8j**	49.9±6.3	**8s**	33.3±7.1
8b	40.6±5.0	**8k**	17.9±8.4	**8t**	22.2±3.7
8c	34.6±9.1	**8l**	33.2±9.5	**8u**	41.0±5.8
8d	36.6±2.3	**8m**	40.1±7.3	**8v**	17.9±5.1
8e	47.2±10.5	**8n**	27.2±1.3	**8w**	34.2±4.4
8f	22.9±8.9	**8o**	40.1±3.8	**8x**	22.4±5.4
8g	32.1±5.5	**8p**	37.3±8.8	Tiadinil	38.1±3.1
8h	39.4±6.8	**8q**	22.9±6.5	Ningnanmycin	57.9±8.4
8i	17.0±11.3	**8r**	26.9±10.1		



8a: R= H **8b**: R= 2-F **8c**: R= 3-F **8d**: R= 4-F **8e**: R= 2-Br **8f**: R= 3-Br **8g**: R= 4-Br

8h: R= 2-Cl **8i**: R= 3-Cl **8j**: R= 4-Cl **8k**: R= 2,4-Cl_2 **8l**: R= 2,5-Cl_2 **8m**: R= 3-CF_3 **8n**: R= 4-CF_3

8o: R= 2-NO_2 **8p**: R= 3-NO_2 **8q**: R= 4-NO_2

8r: R= 3-CH_3 **8s**: R= 4-CH_3 **8t**: R= 4-(t-Bu)

8u: R= 4-OCH_3 **8v**: R= 2-OH **8w**: R= 3,4.5-$(OCH_3)_3$

Scheme1 Synthetic route of the title compounds **8a – 8x**.