Synthesis and study of the antifungal activity of 1-(2-cyanophenyl)-3-heterylureas

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Abstract. In the treatment and prevention of fungal infections of plants, along with fungicides, inducers of systemic plant resistance, also called elicitors, have become particularly important in recent years. In this work, a method was developed for the synthesis of new 3,4-dichloroisothiazol-5-yl and 4-methyl-1,2,3-thiadiazol-5-yl ureas **1**,**2**, containing a 2-cyanophenyl substituent, structurally similar to a known synthetic elicitors isotianil and tiadinil. The protective properties of the obtained compounds on cucumber and pepper leaves infected with *B. cinerea*, as well as their fungicidal properties against *B. cinerea*, were studied. It has been established that disubstituted ureas **1**,**2** exhibit very low fungicidal activity against this fungus, about 11%. At the same time, study on plant leaves showed that compound **2** effectively inhibited the development of gray mold on both cucumber and pepper leaves with an inhibition rate of more than 90%, which was similar to tiadinil. Compound **1** was effective only on cucumber leaves (96.50±0.01%). Isotianil in the experiment showed an average degree of protection – 62.48±1.04% on cucumber leaves and 56.50±1.29% on pepper leaves.

1 Introduction

Combating plant diseases will be a perennial problem that will require all available resources to provide food for the inhabitants of the planet. The UNO estimates that by 2050 the world will need 70% more food due to projected population growth, and this increase will largely need to be offset by improved agricultural efficiency in the limited area of cultivated land [1]. The most widespread diseases of agricultural plants are fungal diseases. They account for more than 80% of all plant diseases [2]. Bacterial and viral infections also cause significant damage to agriculture.

The chemical method of safeguarding plants from phytopathogens holds paramount importance owing to its remarkable efficiency. Treatment with pesticides appears to be a conventional approach in shielding plants from microbial threats. Protective measures using pesticides can save up to 30% or more of the crop yield. Chemicalization of agriculture has become part of the technology for cultivating almost all agricultural crops. However, the use of agrochemicals is associated with threats of environmental pollution – water, air, and soil which leading to a disruption in the ecological balance. This has a negative impact on human

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and animal health [3]. Also, the state of plants under conditions of environmental stress also leads to a decrease in the plant's capacity against infections [4].

Promising directions for protecting plants from diseases are technologies for initiating their disease resistance, based on the implementation of the immune potential of plants. Special significance in this case is the induction of systemic acquired resistance (SAR). SAR is a form of induced resistance with a specific protective signaling pathway that occurs systemically in plants after exposure to a pathogen, as well as after treatment with natural or synthetic compounds called elicitors [5]. Elicitors are compounds that activate the chemical defenses of plants, allowing them to more fully realize their genetic potential. In plants treated with elicitors, different biosynthetic pathways are activated depending on the compound used, as a result of which the plant produces metabolites and copes with the infection itself [6].

The use of elicitors for plant protection and pest control is still in the very early stages of use as a new control method. However, it is known that the use of elicitors allows not only to overcome possible or ongoing contamination of crops, but also to increase their productivity, as well as reduce the consumption of therapeutic and preventive pesticides. All this makes elicitors a promising plant protection product. At the same time, an important advantage of these drugs is their effectiveness in low concentrations (10^{-9}–10^{-6} M) and low toxicity in humans, animals, beneficial microorganisms, etc. [6].

The most important modern synthetic elicitors are described in review [7]. It is worth noting that some of the effective synthetic SAR inducers are derivatives of 1,2,3-thiadiazole and 3,4-dichlorothiazole. The elicitor tiadinil (*N*-(3-chloro-4-methylphenyl)-4-methyl-1,2,3-thiadiazole-5-carboxamide, Nihon Nohyaku Co., Ltd. Japan) was discovered in 2006 [8]. It is available as a commercial product [7] and is widely represented in scientific research [9–11]. Isotianil (3,4-dichloro-N-(2-cyanophenyl)-1,2-thiazole-5-carboxamide) was discovered and developed by Bayer AG (now Bayer CropScience AG) in Germany in 1997 together with the Japanese company Sumitomo Chemical Co.,Ltd. and is highly effective in protecting plants from fungal infections [7,12–13]. There are also known successful examples of targeted synthesis of similar structural analogues of these SAR inducers that show biological properties [14,15].

In the present work, new compounds, 1-(2-cyanophenyl)-3-heterylureas derivatives, structurally similar to isotianil and tiadinil, were developed and their antifungal properties were studied *in vivo* on plant leaves and *in vitro* against *B. cinerea*.

2 Materials and methods

2.1 Materials

Isotianil and tiadinil were purchased from the LEAPChem (China). Carbendazim was purchased from Sigma-Aldrich (USA). *B. cinerea* was purchased from the Russian National Collection of Industrial Microorganisms (Moscow, Russia).

2.2 Methods

2.2.1 Chemical synthesis

^{1}H and ^{13}C NMR spectra were recorded with a Bruker Avance II (Karlsruhe, Germany) spectrometer (400 MHz for ^{1}H, 100 MHz for ^{13}C). Mass spectra were recorded with a Shimadzu GCMS-QP 2010 "Ultra" (Kyoto, Japan) in electron ionization (EI) mode (electron energy 70 eV). The Fourier transform infrared (FT-IR) spectra were obtained using a Bruker

Alpha (ATR, ZnSe) spectrometer (Ettlingen, Germany). Elemental analyses were performed with a Perkin-Elmer 2400Series II CHNS/O analyzer (Shelton, CT USA). Melting points were determined using a Stuart SMP 3 apparatus (Staffordshire, ST15 OSA, UK). The progress of the reactions and the purity of the compounds were monitored by TLC on TLC Silica gel 60 F245 aluminum sheets (Merck KGaA) in an EtOAc-hexane system (1:1 or 1:2).

Synthesis of 3,4-dichloroisothiazol-5-carbonyl azide **3** and 4-methyl-1,2,3-thiadiazole-5-carbonyl azide **4** was carried out using method described early [16].

Acylazide **3** or **4** (1 mmol) was dissolved in dry 1,4-dioxane, 2-cyanoaniline (1 mmol) was added. The reaction mass was refluxed for 4 h. The reaction progress was monitored by TLC. After the reaction was complete, the reaction mass was cooled, and precipitate formed was filtered off and recrystallized from ethanol.

1-(2-Cyanophenyl)-3-(3,4-dichloroisothiazol-5-yl)urea (**1**): white powder, 0.229 g (73 %); m.p. 267–268°C; IR, ν, cm^{-1}: 3337, 3291, 3128, 2297 (C≡N), 1702 (C=O), 1650, 1611, 1590, 1564, 1463, 1447, 1418, 1352, 1325, 1300, 1271, 1250, 1199, 1155, 1049; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.08 (s, 1H, NH), 9.51 (s, 1H, NH), 8.11 (d, J = 8.5 Hz, 1H, H Ar), 7.83 (dd, J = 7.6 Hz, J = 7.8 Hz, 1H, H Ar), 7.73–7.69 (m, 1H, H Ar), 7.29 (t, J = 7.6 Hz, 1H, H Ar); ^{13}C NMR (100 MHz, DMSO-d_6) δ 156.6 (C Het), 151.9 (C=O), 143.9 (C Het), 140.1 (C Ar), 134.3 (CH Ar), 133.4 (CH Ar), 124.5 (CH Ar), 121.5 (CH Ar), 116.5 (C≡N), 102.7 (C Het), 102.5 (C Ar); EI-MS m/z (%): 316 $[M+4]^+$ (3), 314 $[M+2]^+$ (18), 313 $[M+1]^+$ (5), 312 $[M]^+$ (26), 277 (26), 170 (67), 168 (100), 145 (34), 133 (14), 117 (48), 102 (25), 91 (22), 90 (38). Anal. calcd. for $C_{11}H_6Cl_2N_4OS$ (Mr = 313.16): C 42.19, H 1.93, N 17.89, S 10.24; found: C 42.76, H 1.52, N 17.24, S 10.59%.

1-(2-Cyanophenyl)-3-(4-methyl-1,2,3-thiadiazol-5-yl)urea (**2**): beige powder, yield 0.223 g (86 %); m.p. 217–218°C; IR, ν, cm^{-1}: 3245, 3205, 3144, 3061, 3009, 2907, 2231 (C≡N), 1694 (C=O), 1609, 1586, 1562, 1492, 1449, 1396, 1363, 1294, 1251, 1211, 1059; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.77 (br.s, 1H, NH), 9.27 (br.s, 1H, NH), 8.12 (d, J = 8.4 Hz, 1H, H Ar), 7.80 (d, J = 7.7 Hz, 1H, H Ar), 7.69 (t, J = 7.6 Hz, 1H, H Ar), 7.26 (t, J = 7.6 Hz, 1H, H Ar), 2.62 (s, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 151.8 (C=O), 146.6 (C Het), 142.8 (C Het), 140.6 (C Ar), 134.3 (CH Ar), 133.3 (CH Ar), 124.2 (CH Ar), 121.4 (CH Ar), 116.6 (C≡N), 102.4 (C Ar), 11.8 (CH_3); EI-MS m/z (%): 259 $[M]^+$ (2), 231 $[M–N_2]^+$ (99), 198 (48), 145 (57), 118 (79), 117 (65), 91 (54), 90 (54), 88 (60), 87 (100), 70 (22), 60 (52), 59 (43), 42 (45), 39 (21). Anal. calcd. for $C_{11}H_9N_5OS$ (Mr = 259.29): C 50.96, H 3.50, N 27.01, S 12.36; found: C 50.82, H 3.59, N 27.58, S 12.87%.

*2.2.2 Determination of protective activity of compounds **1** and **2** on plant leaves*

The *in vivo* protective activity of compounds **1** and **2** against *B. cinerea* was evaluated at a concentration of 200 μg/mL in 0.1% aqueous DMSO solution, according to previously reported method [17]. Commercial elicitors tiadinil and isotianil were chosen as positive controls. The negative control was sprayed with sterile water containing 0.1% of DMSO. Cucumber and pepper plants were grown in pots to the 5-leaf stage. Leaves were cut and sprayed with solution of test compound and placed in 10-mm sterile Petri dishes on 2 pieces of filter paper. Then, 5 mL of sterile water was added to maintain humidity. After 24 h, discs of mycelia were placed on each side of the leaf at an equal distance from the median vein. Inoculated leaves were placed at 25°C with a daily 16-h light period and 70% humidity in the Binger climate chamber for disease development. After 5 days of inoculation, disease spot area was measured using ImageJ 1.52u program (NIH, USA) and protection efficacy (%) was calculated according to the above formula:

$$I(\%) = [(S_1 - S_2) / S_1] \times 100 \quad (1)$$

Where S_1 is the area of the necrotic spot of the negative control, cm^2; S_2 – area of necrotic spot on the leaf treated with the test compound, cm^2.

All the experiments were carried out in triplicate. The statistical data analysis was carried out using the Statistica 13 program (StarSoft, USA) and confidence interval was calculated based on the Student *t*-distribution at confidence level of 95%.

2.2.3. Fungicidal activity in vitro

The fungicidal activity of compounds **1-2** was studied *in vitro* according to a previously published method [17] against the following phytopathogenic fungus *Botrytis cinerea*. The fungicidal properties of target compounds and carbendazim (positive control) were studied at a concentration of 50 μg/ml. The solutions of the compounds were prepared at a concentration of 1 mg/mL by dissolving of 5 mg of the compound in 1 mL of DMSO accompanied by an addition of 9 mL of water. A total of 1 mL of the tested solutions was added in Petri dishes containing 9 mL of the heated culture medium and was homogeneously mixed. Uniform fungal discs (4 mm diameter) were aseptically cut from a 7-day old culture of the test fungus, using a sterile cork borer. Discs of mycelia were placed on the center of Petri dishes containing room temperature culture medium. The negative control was prepared with culture medium and DMSO only. The fungi were incubated 72 h at 25 °C and the diameter of the fungal colonies was measured after incubation. Inhibition of the pathogen development was determined according to the formula of Royse and Ries [18]:

$$I(\%) = [(C - T)/(C - 4\ mm)] \times 100 \quad (2)$$

Where I (%) is the degree of inhibition of mycelial growth, T (mm) is the mean value of the diameter of the colonies in the presence of a given concentration of each compound and C (mm) is the mean diameter of the colonies in the absence of the compound under the same conditions. All the experiments were carried out in triplicate. The standard deviation was calculated.

The obtained data of fungicidal activity for the studied compounds are shown in **Table 2**.

3 Results and discussions

Similar structural analogs of the known inducers of systemic plant resistance isotianil and tiadinil 3,4-dichloroisothiazol-5-yl and 4-methyl-1,2,3-thiadiazol-5-yl ureas **1** and **2** were first synthesized by the reaction of acylazides **3** and **4**, respectively, with 2-cyanoaniline (Scheme 1). Compound **1** differs from isotianil only by the NH bridge in the structure. Urea **2** contains an *N*-2-cyanophenylcarboxamide fragment in its structure, like isotianil, and also 4-methyl-1,2,3-thiadiazol-5-yl, like tiadinil.

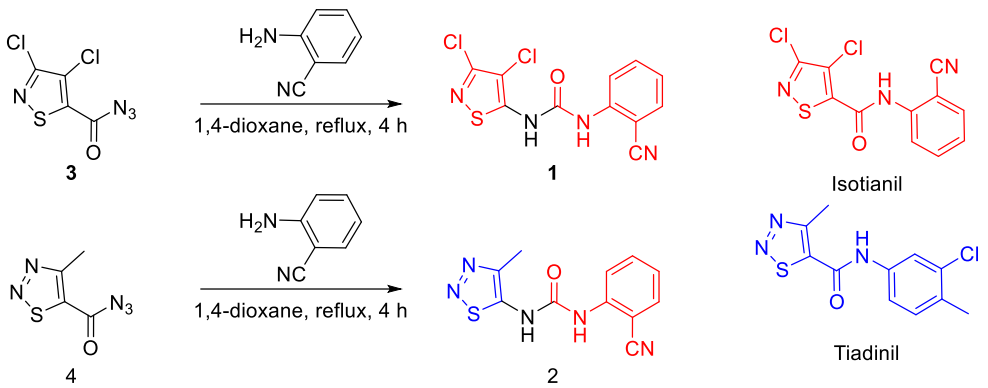

Fig. 1. Synthesis of the target 1-(2-cyanophenyl)-3-heterylureas **1**,**2** and the structure of the known elicitors isotianil and tiadinil.

The study of the antifungal properties of compounds **1**,**2**, as well as isotianil and tiadinil elicitors against *B. cinerea* (causative agent of gray mold) on the leaves of cucumber and pepper plants showed promising results (**Table 1** and **figures 2,3**). It was shown that synthesized ureas **1**,**2** and tiadinil inhibit the development of cucumber leaf disease by more than 90% compared to the control. However, on pepper leaves, only compound **2** and tiadinil showed high protective ability, also inhibiting the disease by more than 90%. 3,4-Dichloroisothiazol-5-ylurea **1** showed the least protection on pepper leaves, reducing leaf damage by only 21.02±1.63%. Isotianil in both experiments showed average activity inhibiting the disease of cucumber and pepper leaves by 62.48±1.04 and 56.50±1.29%, respectively.

It is known that, depending on the plant species and the elicitor, it takes a certain period to create SAR. It is also known that some pathogens do not respond to the use of certain resistance inducers, or even increased plant damage is observed [19,20]. It may be necessary to re-treat the leaves to improve the protective properties of isotianil.

Table 1. Determination of protective activity of compounds against *B. cinerea* on plant leaves.

Treatment	Cucumber leaves		Pepper leaves	
	Infection area, cm²	**Inhibition degree, (I ± SD%)**	**Infection area, cm²**	**Inhibition degree, (I ± SD%)**
1	0.69±0.23	96.50±0.01	18.82±2.06	21.02±1.63
2	1.40±0.25	92.91±0.02	1.20±0.46	94.96±0.02
Tiadinil	1.84±0.34	90.70±0.03	2.26±0.43	90.53±0.04
Isotianil	7.41±2.77	62.48±1.04	10.37±2.97	56.50±1.29
Control	19.74±1.90	–	23.83±1.67	–

SD—standard deviation, I = 100—active compound, I = 0—not active compound.

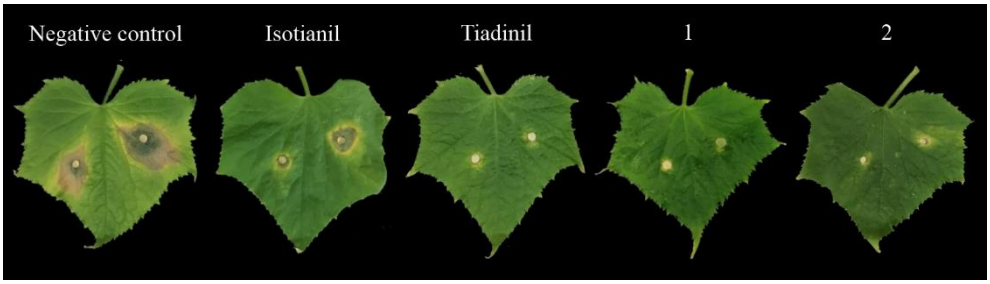

Fig. 2. Representative samples of cucumber leaves after treatment and infection by *B. cinerea*.

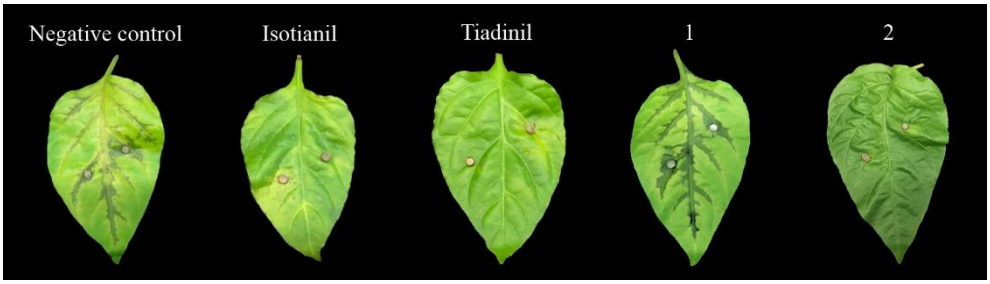

Fig. 3. Representative samples of pepper leaves after treatment and infection by *B. cinerea*.

Studies show that elicitors do not act on the pathogen itself, but protect the plant by activating its natural defense systems [21]. To test the antifungal properties of the synthesized compounds **1**,**2**, their effect on the growth of fungal colonies *in vitro* was studied. The results are presented in **Table 2**. The well-known fungicide carbendazim was used as a positive control.

Table 2. Degree of fungal growth inhibition for compounds **1**,**2** at a concentration of 50 μg/mL against *B. cinerea*.

Compound	Degree of inhibition of mycelial growth (I±SD%)
1	11.21±1.20
2	11.28±0.34
Carbendazim	100 ± 0.00

SD—standard deviation, I = 100—active compound, I = 0—not active compound.

Unlike carbendazim (I=100%), ureas **1**,**2** showed very low fungicidal activity – 11.21±1.20% and 11.28±0.34%, respectively. This may provide evidence that these compounds act as elicitors rather than fungicides. However, to confirm this theory, additional studies using biochemical, immunological and molecular biological methods are needed.

4 Conclusions

The use of elicitors is one of the new and actively developing areas in protecting plants from microbial pathogens. In this study, a method for the synthesis of new 3,4-dichloroisothiazol-5-yl and 4-methyl-1,2,3-thiadiazol-5-yl ureas **1** and **2** was developed and their potency of use as an elicitor was demonstrated. Particularly, interesting from this point of view is 4-methyl-1,2,3-thiadiazol-5-ylurea **2**, which showed promising protective properties on the leaves of both cucumber and pepper plants against infection by *B. cinerea*. To develop plant protection products based on the obtained compounds **1**,**2**, further comprehensive research of their biological effects against other phytopathogens and various plants is necessary. It is also

necessary to study their toxicity in humans, animals, soil microflora, mechanism of biological action, resistance, etc.

This research was supported by the Russian Science Foundation and Government of Sverdlovsk region, Joint Grant No 22-26-20124, https://rscf.ru/en/project/22-26-20124/ (accessed on 30 October 2023).

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