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Mono- and bis-dipicolinic acid heterocyclic derivatives – thiosemicarbazides, triazoles, oxadiazoles and thiazolidinones as antifungal and antioxidant agents

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Abstract: A series of dipicolinic acid derivatives was synthesized and investigated for antimicrobial and antioxidant activity. Mono and bis derivatives of ethyl dipicolinate were utilized as starting materials for synthesis of mono- and bis-hydrazides. Thiosemicarbazides were obtained by reaction of hydrazides with isothiocyanates and cyclized into triazoles, thiadiazoles, oxadiazoles and thiazolidinones. Some of these products, especially those incorporating a thiazolidinone moiety in their structure, are excellent antioxidants, DPPH scavengers and antifungal agents.

Keywords: antifungal; antioxidant activity; dipicolinic acid; oxadiazole; thiadiazole; thiazolidinone; triazole.

Introduction

Dipicolinic acid (DPA) is a unique constituent of endospores of *Bacillus* and *Clostridium* genuses [1] and is also produced and secreted by certain *Penicillium* strains and by several entomopathogenic fungi [2]. DPA and its derivatives show various biological activities including significant antimicrobial [3] and antioxidant properties [4]. As a strong complexing agent DPA is a potent metal-chelator functioning as a multidentate ligand [5] that inhibits lipid peroxidation and protects glutathione reductase from the copper-dependent inactivation [6]. A structural combination of DPA core with other heterocyclic compounds has already proven to be an excellent tool for gaining antimicrobial and antioxidant activity [7, 8]. In this work, thiadiazole, triazole, thiazolidinone and oxadiazole moieties were combined with the DPA core in order to achieve the expected potent antimicrobial and/or antioxidant activity. Diverse biological and/or antioxidant properties of 1,3,4-thiadiazoles [9–14], triazoles [11, 13–15], thiazolidinones [16–21], and oxadiazoles [22] have been documented. Only a slight change in structural characteristics can have a great effect on antifungal and antioxidant activity [4, 17]. To date, derivatives of dipicolinic acid were described by Milway in 2003 [23] and in our previous work on Schiff bases [4].

Results and discussion

The synthesis of the target compounds was carried out as outlined in Schemes 1–3. All products were purified and characterized by TLC, MS, ^{1}H NMR and elemental analysis. Preparation of starting compounds 6-methoxycarbonyl-2-pyridinecarboxylic acid hydrazide (**1**) and 2,6-pyridinedicarboxylic acid bis-hydrazide (**2**) was described in our previous work [4]. Thiosemicarbazides were prepared by refluxing mono-hydrazide or bis-hydrazide with the corresponding isothiocyanate in 1 : 1 ratio for mono derivatives and 1 : 2 ratio for bis derivatives in ethanol (Scheme 1).

Thiosemicarbazides exhibit characteristic ^{1}H NMR peaks for NH groups and the difference between mono and bis derivatives is clearly visible in the presence of the characteristic peak at δ 3.93 corresponding to $-OCH_3$ group of mono derivatives. Conventional cyclization of thiosemicarbazides yields heterocyclic compounds such as thiadiazole, oxadiazole, triazole and thiazolidinone. Thus, cyclization of thiosemicarbazides in the presence of an excess of sulfuric acid gave thiadiazoles. This reaction was succesfully performed for bis-thiosemicarbazides

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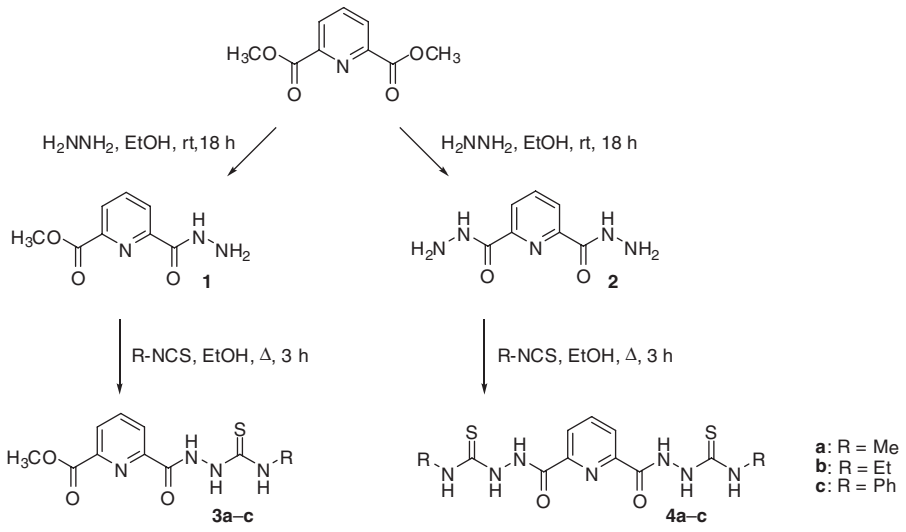
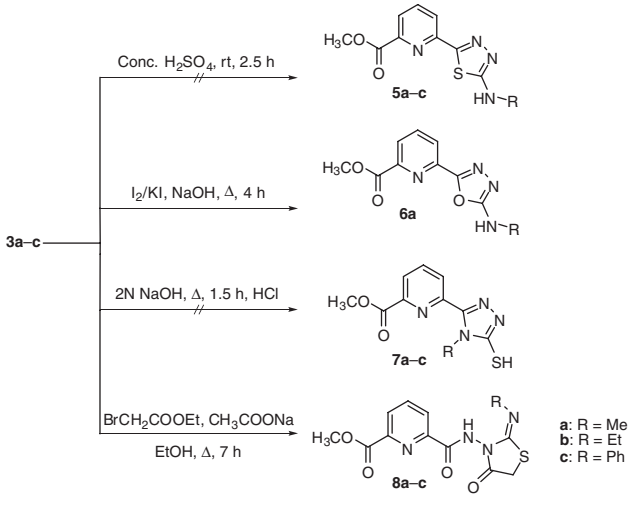


Scheme 1 Synthetic route to compounds **1–4**.

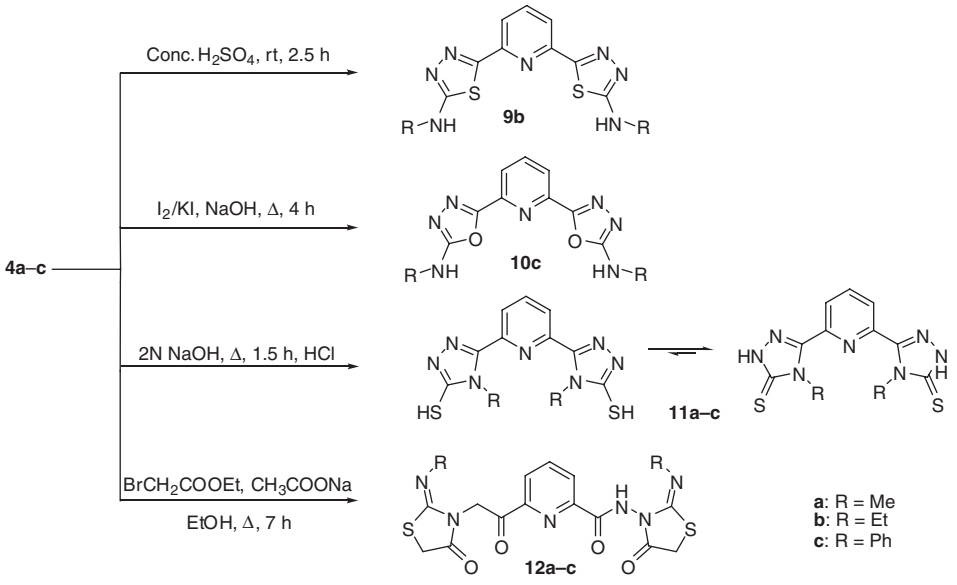
only, since no expected products for mono derivatives were obtained, due to hydrolysis of the ester group by a strong acid. Bis derivatives of these compounds were published by Foroughifar and co-workers in 2014 [24]. Upon treatment of thiosemicarbazides with I_2/KI system in NaOH solution, oxadiazoles were formed showing NH signals in the ^{1}H NMR spectra, in addition to signals for groups characteristic for each substituent depending on isothiocyanate used in thiosemicarbazide synthesis. Oxadiazole synthesis using I_2/KI system was successful for only two derivatives, monosubstituted with the methyl and disubstituted with phenyl groups. Treatment of thiosemicarbazides with NaOH resulted in cyclization to triazoles. The synthesized triazoles show characteristic peaks for NH group (triazole ring) at δ 14.15–14.27, indicating that they exist mainly in a thione form rather than with a thiol function. Only disubstituted derivatives of triazoles were synthesized [24], since the mono derivatives underwent hydrolysis of the methoxycarbonyl group. Refluxing monosubstituted and disubstituted thiosemicarbazides with ethyl bromoacetate in ethanol in the presence of sodium acetate yielded corresponding thiazolidinones. These compounds show a characteristic signal for the CH_2 group of the thiazolidinone, which is not visible in the spectra of the corresponding thiosemicarbazides.



Scheme 2 Synthetic route to compounds **6a** and **8a–c**.

The synthesized compounds were assayed for 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity (Figure 1). As can be seen, compound **3c** is an excellent scavenger, with activity comparable to that of ascorbic acid. The overall data show a predominant antioxidant activity of thiosemicarbazides compared to other synthesized derivatives of DPA. Also the influence of different substituents is obvious, since in almost all compounds the phenyl substitution results in a better DPPH radical scavenging activity in comparison to the alkyl substitution. The same trend has also been observed in our previous work [4, 17, 25]. Triazoles **11a–c** show moderate DPPH scavenging activity that are better than activities of thiazolidinones, oxadiazoles and thiadiazoles.

Antibacterial properties of the synthesized compounds were studied against four bacteria, namely two Gram-positive (*Bacillus subtilis* and *Staphylococcus aureus*) and two Gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*) species. None of the compounds shows noticable antimicrobial activity comparable to antibiotic amikacin, an aminoglycoside that is highly active against most Gram-negative bacteria including many



Scheme 3 Synthetic route to compounds **9–12**.

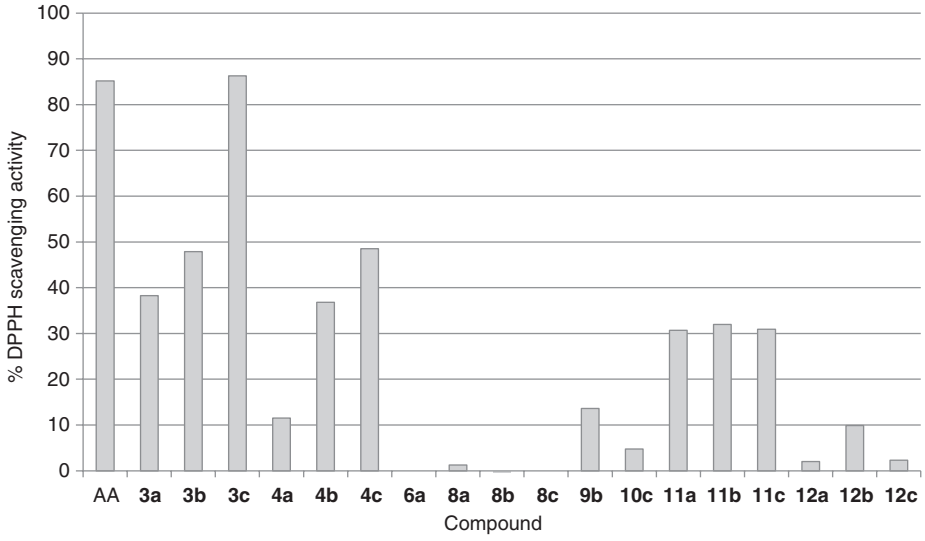


Figure 1 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of dipicolinic acid derivatives. AA is ascorbic acid taken as a reference.

gentamicin-resistant strains [26]. Amikacin is markedly active against antibiotic-resistant clinical isolates [27].

Antifungal activity of the compounds was assayed against four fungal strains, namely *Aspergillus flavus*, *Aspergillus ochraceus*, *Fusarium graminearum* and *Fusarium verticilioides*. As in our previous work [17], *F. graminearum* was found to be highly susceptible to the tested compounds but most of the compounds exhibit impressive antifungal activity against all tested fungi (Table 1).

In general, *Fusarium* strains are less resistant than *Aspergilus* strains (not shown). *Aspergillus flavus* is the most resistant to tested compounds and the best antifungal agent against this mold is compound **10b**. *Aspergillus ochraceus* is the most affected by **3b**, **11b**, **12b**, all of them incorporating different ring systems and possessing the ethyl group in their structure. For *Fusarium* strains almost all compounds exhibit significant antifungal activity at given concentrations. The difference between sensitivity of *Aspergillus* and *Fusarium* strains may be due to their differences in cell structure. Since *Aspergillus* spp. is mainly human pathogen [28] and according to phylogenetic tree is more closely related to *Penicilium* spp. [29, 30] that can produce DPA [2], it is expected to have higher resistance to DPA derivatives (as confirmed). On the other hand,

Table 1 Antifungal activity of dipicolinic acid derivatives expressed as MIC_{50}.

Test compound	MIC_{50} (μg/mL)			
	A. flavus	*A. ochraceus*	*F. graminearum*	*F. verticilioides*
3a	1	0.1	< 0.1	< 0.1
3b	0.1	< 0.1	< 0.1	< 0.1
3c	1	0.1	0.1	0.1
4a	10	0.1	< 0.1	< 0.1
4b	NA	NA	0.1	0.1
4c	0.1	0.1	< 0.1	0.1
6a	100	0.1	< 0.1	0.1
8a	0.1	0.1	0.1	0.01
8b	10	10	0.1	10
8c	0.1	0.1	0.1	0.1
9b	10	NA	0.1	0.1
11a	0.1	1	< 0.1	< 0.1
11b	< 0.1	< 0.1	< 0.1	< 0.1
11c	10	10	0.1	0.1
12a	0.1	10	0.1	0.1
12b	0.1	< 0.1	< 0.1	< 0.1
12c	1	0.1	0.1	< 0.1

Fusarium spp. is mainly a plant pathogen/saprophyte [31] and, therefore, more susceptible to DPA. *Aspergillus* spp. is also charaterized by a great number of cell wall associated enzymes that can degrade (hydrolyze) synthesized compounds due to their unspecific activity [32, 33]. On the other hand, *Fusarium* spp. possess a cell wall associated unspecific hydrolases [34] that are mainly intended to increase their virulence toward plants [31, 34]. This is a great indicator for design and synthesis of some future antifungal agents, especially against *Aspergillus* strains, since the triazole moiety substituted with the ethyl group has a significant impact on this activity against both *A. flavus* and *A. ochraceus*. Triazole derivatives may be regarded as a new class of antifungal agents which can inhibit fungi by blocking the biosynthesis of certain fungal lipids, especially, ergosterol in cell membranes [35]. The differences between antifungal and antibacterial activities are probably caused by different mechanisms of action. The structures of fungi and bacteria differ in very significant ways. For example, many antibacterial agents inhibit steps important for the formation of peptidoglycan, the essential component of the bacterial cell wall. In contrast, most antifungal compounds target either the formation or the function of ergosterol, an important component of the fungal cell membrane. Bacteria employ an extensive repertoire of plasmids, transposons, and bacteriophages to facilitate the exchange of resistance and virulence determinants among and between species. Conversely, antifungal resistance generally involves the emergence of naturally resistant species [36].

Conclusions

Dipicolinic acid derivatives were synthesized and investigated for antimicrobial and antioxidant activity. Some compounds show excellent antioxidant activity, the thiosemicarbazide **3c**, in particular. Thiosemicarbazide **3b** possess good antioxidant activity, but it is also an excellent antifungal agent against all four investigated fungi strains. Data collected in this research indicate that thiosemicarbazides could be used as a unique antifungal and antioxidant agents.

Experimental

Melting points were determined on an Electrothermal capillary apparatus. The elemental analysis for C, H and N was done on a Perkin-Elmer CHNS/O analyzer 2400 Series II. ^{1}H NMR spectra were recorded in DMSO-d_6 at 293 K on a Bruker Avance 600 MHz spectrometer. The MS spectra were recorded on an LC-MS/MS API 2000 instrument using electrospray ionization (ESI). The spectra were scanned in both positive and negative ion modes. The absorbance was measured on a UV visible spectrophotometer Helios γ. Microplates were read on a Sunrise absorbance reader. Incubation was carried in an Aqualytic AL 500-8 incubator.

Preparation of thiosemicarbazides 3a–c and 4a–c

A mixture of methyl 2-carbazoylpyridin-6-carboxylate (**1**, 10 mmol) or 2,6-bis-carbazoylpyridine (**2**, 10 mmol) and methyl, ethyl or phenyl isothiocyanate (10 mmol for **1** and 20 mmol for **2**) in absolute ethanol

(50 mL) was heated under reflux for 3 h. Progress of the reaction was monitored by thin layer chromatography (TLC). Crude product, **3a–c** or **4a–c**, was filtered and crystallized from ethanol [17].

Methyl 6-(4-methylthiosemicarbazide-1-yl-carbonyl)pyridin-2-carboxylate (3a) This compound was obtained from **1** and methyl isothiocyanate; yield 90%; mp 210°C; ^{1}H NMR: δ 2.85 (m, 3H, CH_3), 3.93 (s, 3H, OCH_3), 8.23–8.25 (m, 3H, py-H); 8.01 (d, 1H, J = 4.1 Hz, NH), 9.39 (brs, 1H, NH), 10.36 (brs, 1H, NH). Anal. Calcd for $C_{10}H_{12}N_4O_3S$ (268.06): C, 44.77; H, 4.51; N, 20.88. Found: C, 44.92; H, 4.68; N, 19.97.

Methyl 6-(4-ethylthiosemicarbazide-1-yl-carbonyl)pyridin-2-carboxylate (3b) This compound was obtained from **1** and ethyl isothiocyanate; yield 92%; mp 208°C; ^{1}H NMR: δ 1.07 (m, 3H, CH_3), 3.46 (m, 2H, CH_2), 3.94 (s, 3H, OCH_3), 8.08 (t, 1H, J = 5.5 Hz, NH), 8.24–8.26 (3H, py-H), 9.36 (brs, 1H, NH), 10.36 (brs, 1H, NH); MS: *m/z* 281 $[M–H]^-$, (M = 282.3). Anal. Calcd for $C_{11}H_{14}N_4O_3S$ (282.08): C, 46.80; H, 5.00; N, 19.85. Found: C, 46.02; H, 5.26; N, 19.38.

Methyl 6-(4-phenylthiosemicarbazide-1-yl-carbonyl)pyridin-2-carboxylate (3c) This compound was obtained from **1** and phenyl isothiocyanate; yield 80%; mp 162–164°C; ^{1}H NMR: δ 3.94 (m, 3H, OCH_3), 7.14–7.49 (5H, ArH), 8.24–8.26 (3H, py-H), 9.78 (brs, 2H, 2NH), 10.57 (s, 1H, NH); MS: *m/z* 328.9 $[M–H]^-$, (M = 330.3). Anal. Calcd for $C_{15}H_{14}N_4O_3S$ (330.08): C, 54.54; H, 4.27; N, 16.96. Found: C, 53.1; H, 4.32; N, 16.54.

2,6-Bis(4-methylthiosemicarbazide-1-yl-carbonyl)pyridine (4a) This compound was obtained from **2** and methyl isothiocyanate; yield 74%; mp 202–207°C; ^{1}H NMR: δ 2.70 (d, 6H, J = 4.1 Hz, CH_3), 8.14 (m, 2H, NH), 8.16 (3H, py-H), 9.53 (s, 2H, NH), 11.00 (s, 2H, NH); MS: *m/z* 339.8 $[M–H]^-$, (M = 341.4). Anal. Calcd for $C_{11}H_{15}N_7O_2S_2$ (341.07): C, 38.70; H, 4.43; N, 28.72. Found: C, 38.85; H, 4.95; N, 28.62.

2,6-Bis(4-ethylthiosemicarbazide-1-yl-carbonyl)pyridine (4b) This compound was obtained from **2** and ethyl isothiocyanate; yield 78%; mp 219–220°C; ^{1}H NMR: δ 1.05 (s, 6H, CH_3), 3.43–3.48 (m, 4H, CH_2), 8.03–8.07 (m, 2H, NH), 8.22–8.25 (m, 3H, py-H), 9.33 (brs, 2H, NH), 10.32 (m, 2H, NH); MS: *m/z* 368.1 $[M–H]^-$, (M = 369.4). Anal. Calcd for $C_{13}H_{19}N_7O_2S_2$ (369.10): C, 42.26; H, 5.18; N, 26.54. Found: C, 42.75; H, 5.28; N, 26.75.

2,6-Bis(4-phenylthiosemicarbazide-1-yl-carbonyl)pyridine (4c) This compound was obtained from **2** and phenyl isothiocyanate; yield 68%; mp 234°C (lit mp 245–247°C [8]); ^{1}H NMR: δ 7.13–7.47 (m, 10H, arom), 8.23–8.25 (m, 3H, py-H), 9.85–9.91 (4H, NH), 11.25 (brs, 2H, NH); MS: *m/z* 464.00 $[M–H]^-$, (M = 465.5). Anal. Calcd for $C_{21}H_{19}N_7O_2S_2$ (465.10): C, 54.18; H, 4.11; N, 21.06. Found: C, 53.65; H, 4.23; N, 20.27.

2,6-Bis(5-ethylamino-1,3,4-thiadiazol-2-yl) pyridine (9b)

A solution of compound **4b** (5 mmol) in concentrated sulfuric acid (5 mL) was kept at room temperature for 2.5 h and then poured over ice water. The precipitated product **9b** was filtered and crystalized from *N,N*-dimethylformamide (DMF) [37]: yield 38%; mp 287°C; ^{1}H NMR: δ 1.25 (s, 6H, CH_3), 3.39–3.46 (m, 4H, CH_2), 7.85 (brs, H, NH), 7.86–8.11 (m, 2H, py-H); MS: *m/z* 334.2 $[M+H]^+$, (M = 333.4).

Preparation of oxadiazoles 6a–c from 3a–c and 10c from 4c

A solution of compound **3a–c** or **4c** in EtOH (20 mL) was treated with I_2 (500 mg), KI (640 mg in 20 mL of water) and NaOH (4N, 2 mL) and the mixture was heated under mild reflux for 4.5 h [37]. Progress of the reaction was monitored by TLC. Concentration to half a volume resulted in precipitation **6a–c** or **10c**. The product was filtered and crystallized from EtOH. The representative data for **6a** and **10c** are given below.

Methyl 6-(5-methylamino-1,3,4-oxadiazol-2-yl)pyridine-2-carboxylate (6a) Yield 76%; mp > 300°C; ^{1}H NMR: δ 3.83 (s, 3H, CH_3), 4.07 (s, 3H, OCH_3), 7.92–7.98 (m, 3H, py-H); 8.61 (m, 1H, NH); MS: *m/z* 234.90 $[M–H]^-$, (M = 234.2).

5,5′-Bis(phenylamino-1,3,4-oxadiazol-2-yl)pyridine (10c) Yield 48%; mp 298°C (lit mp 303–305°C, [8]); ^{1}H NMR: δ 7.36–7.67 (10H, arom), 8.22 (3H, py-H), 10.93 (s, 2H, NH); MS: *m/z* 396.10 $[M–H]^+$, (M = 397.40).

Preparations of triazoles 11a–c from 4a–c

A solution of **4a–c** (10 mmol) in aqueous NaOH (2N, 10 mL) was heated under mild reflux for 1.5 h, then cooled, poured over ice water and the mixture was acidified with diluted hydrochloric acid [37]. Product **11a–c** was separated by filtration and crystallized from DMF/water.

2,6-Bis(4-methyl-4*H*-5-sulfanyl-1,2,4-triazol-3-yl)pyridine (11a) Yield 75%; mp > 300°C; ^{1}H NMR: δ 3.82 (s, 6H, CH_3), 8.13–8.28 (m, 3H, py-H); 14.15 (br.s, 2H, NH); MS: *m/z* 304.20 $[M–H]^-$, (M = 305.4). Anal. Calcd for $C_{11}H_{11}N_7S_2$ (305.05): C, 43.26; H, 3.63; N, 32.11. Found: C, 43.48; H, 3.91; N, 32.00.

2,6-Bis(4-ethyl-4*H*-5-sulfanyl-1,2,4-triazol-3-yl)pyridine (11b) Yield 72%; mp > 300°C; ^{1}H NMR: δ 1.15–1.20 (t, 6H, J = 7.0 Hz, CH_3), 4.41–4.48 (m, 4H, CH_2), 8.12–8.24 (m, 3H, py-H); 14.24 (br.s, 2H, NH); MS: *m/z* 332.10 $[M–H]^-$, (M = 333.4). Anal. Calcd for $C_{13}H_{15}N_7S_2$ (333.08): C, 46.83; H, 4.53; N, 29.41. Found: C, 46,34; H, 4.51; N, 29.15.

2,6-Bis(4-phenyl-4*H*-5-sulfanyl-1,2,4-triazole-3-yl)pyridine (11c) Yield 70%; mp > 300°C (lit mp 338–340°C, [8]); ^{1}H NMR: δ 7.03–7.45 (m, 10H, arom), 7.74–8.02 (3H, py-H); 14.17 (br.s, 2H, NH); MS: *m/z* 428.00 $[M–H]^-$, (M = 429.5). Anal. Calcd for $C_{21}H_{15}N_7S_2$ (429.08): C, 58.72; H, 3.52; N, 22.83. Found: C, 58.95; H, 3.71; N, 21.57.

Preparations of thiazolidinones 8a–c from 3a–c and 12a–c from 4a–c

A mixture of thiosemicarbazide **3a–c** or **4a–c** (10 mmol), ethyl bromoacetate (11 mmol for **3a–c** or 22 mmol for **4a–c**) and anhydrous sodium acetate (40 mmol) in absolute ethanol (30 mL) was heated under reflux for 7–10 h [17]. Then the mixture was cooled, poured onto ice and water and the resultant solid was filtered, dried and crystallized from EtOH.

Methyl 6-[(2-methylimino-4-oxothiazolidin-3-yl)carbamoyl]pyridine-2-carboxylate (8a)

Yield 82%; mp 212°C; ^{1}H NMR: δ 3.17–3.30 (s, 3H, CH_3), 3.95 (s, 3H, OCH_3), 4.12 (s, 2H, CH_2), 8.23–8.26 (m, 3H, py-H); 10.57 (s, H, NH); MS:

m/z 307.10 $[M-H]^-$, (M = 308.3). Anal. Calcd for $C_{12}H_{12}N_4O_4S$ (308.06): C, 46.75; H, 3.92; N, 18.17. Found: C, 46.73; H, 3.85; N, 17.9.

Methyl 6-[(2-ethylimino-4-oxothiazolidin-3-yl)carbamoyl]pyridine-2-carboxylate (8b)

Yield 70%; mp > 300°C; ^{1}H NMR: δ 1.16–1.34 (t, 3H, J = 7.0 Hz, CH_3), 3.74–3.79 (m, 2H, CH_2), 3.93 (s, 3H, OCH_3), 4.03 (s, 2H, CH_2), 8.15–8.28 (m, 3H, py-H); 10.64 (s, H, NH); MS: *m/z* 321.20 [M–H], (M = 322.34). Anal. Calcd for $C_{13}H_{14}N_4O_4S$ (322.07): C, 48.44; H, 4.38; N, 17.38. Found: C, 47.90; H, 4.43; N, 17.81.

Methyl 6-[(2-phenylimino-4-oxothiazolidin-3-yl)carbamoyl]pyridine-2-carboxylate (8c)

Yield 88%; mp 235°C; ^{1}H NMR: δ 3.94 (s, 3H, OCH_3), 4.27 (m, 2H, CH_2), 7.39–7.56 (5H, arom), 8.19–8.24 (m, 3H, py-H); 10.54 (br.s, H, NH); MS: *m/z* 369.10 $[M-H]^-$, (M = 370.1); Anal. Calcd for $C_{17}H_{14}N_4O_4S$ (370.07): C, 55.13; H, 3.81; N, 15.13. Found: C, 55.19; H, 3.71; N, 15.21.

2,6-Bis[(2-methylimino-4-oxothiazolidin-3-yl)carbamoyl]pyridine (12a) Yield 77%; mp > 300°C; ^{1}H NMR: δ 3.21 (s, 6H, CH_3), 4.09 (s, 4H, CH_2), 8.21 (m, 3H, py-H); 11.60 (brs, 2H, NH); MS: *m/z* 420.0 $[M-H]^-$, (M = 421.4). Anal. Calcd for $C_{15}H_{15}N_7O_4S_2$ (421.1): C, 42.75; H, 3.59; N, 23.26. Found: C, 42.49; H, 3.56; N, 22.66.

2,6-Bis[(2-ethylimino-4-oxothiazolidin-3-yl)carbamoyl]pyridine (12b) Yield 70%, mp 214–217°C; ^{1}H NMR: δ 1.20–1.24 (t, 6H, *J* = 7.0 Hz, CH_3), 3.77–3.83 (m, 4H, CH_2), 4.08 (s, 4H, CH_2), 8.21 (3H, py-H); 11.61 (s, 2H, NH); MS: *m/z* 448.20 $[M-H^+]^-$, (M = 449.50). Anal. Calcd for $C_{17}H_{19}N_7O_4S_2$ (449.09): C, 45.42; H, 4.26; N, 21.81. Found: C, 44.92; H, 4.26; N, 22.38.

2,6-Bis[(2-phenylimino-4-oxothiazolidin-3-yl)carbamoyl]pyridine (12c) Yield 58%; mp 202°C; ^{1}H NMR: δ 4.24–4.34 (m, 4H, CH_2), 6.88–7.56 (10H, arom), 8.28–8.38 (3H, py-H); 11.88 (s, 2H, NH); MS: *m/z* 544.0 $[M-H]^-$, (M = 545.6). Anal. Calcd for $C_{25}H_{19}N_7O_4S_2$ (545.09): C, 55.04; H, 3.51; N, 17.97. Found: C, 55.04; H, 3.51; N, 17.63.

DPPH scavenging activity

Determination of antioxidant activity was performed according to the procedure described in our previous work [17]. Briefly, 750 μL of 0.2 mM dimethyl sulfoxide (DMSO) solution of the compound was added to 0.2 mM DMSO solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, resulting in 0.1 mM solution of tested compound. Ascorbic acid (AA) was used as a reference. After 30 min of incubation the absorbance was read at 517 nm and the scavenging activity was calculated as described previously [17].

Antibacterial activity

The antibacterial activities of DPA derivatives were evaluated against four test bacteria strains. Two Gram-positive *Bacillus subtilis* and *Staphylococcus aureus*, and two Gram-negative *Escherichia coli* and *Pseudomonas aeruginosa* were used. The four bacteria were isolates from various clinical specimens obtained from Microbiology Service of the Public Health Institute of Osijek-Baranja County, Croatia. *Bacillus subtilis* and *Escherichia coli* were selected as two popular laboratory model organisms representing Gram-positive and Gram-negative bacteria, respectively. *Staphylococcus aureus* and *Pseudomonas aeruginosa* were selected as human pathogens representing Gram-positive and Gram-negative bacteria, respectively. The antibacterial activity was assessed in terms of minimum inhibitory concentrations (MICs) by a modified broth microdilution method [38]. Broth microdilution tests were performed with 96 sterile flat-bottom microtiter plates (Ratiolab, Dreieich, Germany). Compounds dissolved in DMSO and one hundred microliters of Mueller-Hinton Broth (MHB) (Cultimed) were prepared in 96 well micro-trays. Antimicrobials were serially diluted (512 to 1 μg/mL) in MHB. The inoculum was prepared by making a MHB suspension of colonies from a 24 h Mueller-Hinton Agar (MHA) plate culture of the microorganisms. Each well was inoculated with 300×10^3 bacteria (density of the bacterial suspension at 0.5 McFarland scale, which is 150×10^6 CFU/mL. Amikacin sulfate (AMCK) was co-assayed as positive control, and DMSO was used as negative control. Control samples (positive and negative) were incubated under the same conditions. After incubation at 37°C for 24 h with 5% CO_2 and 50% humidity, the trays were examined for the growth of the test microorganisms with the unaided eye. The MIC value was defined as the lowest concentration of compound at which there was no visual turbidity due to microbial growth. All assays were performed in duplicate.

Antifungal activity

The method was previously described in detail [17] and was performed in accordance with the guidelines detailed in [39]. Fungi strains used in these experiments were *Aspergillus flavus* (NRRL 3251), *Aspergillus ochraceus* (CBS 589.68), *Fusarium graminearum* (CBS 110.250) and *Fusarium verticillioides* (CBS 119.825). These species were selected as major producers of mycotoxins and food contaminants [40].

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