Journal of the Iranian Chemical Society
https://doi.org/10.1007/s13738-017-1283-x

ORIGINAL PAPER

Design, synthesis, structure elucidation and in vitro antiviral and antimicrobial evaluation

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Received: 21 June 2017 / Accepted: 23 December 2017

Abstract
In this study, we described the synthesis of the derivatives of thiosemicarbazide, dicarboximide, 1,2,4-triazole-5-thione and 4-oxo-1,3-thiazolidine. Two different dicarboxylic acid anhydrides reacted with 4-substituted-3-thiosemicarbazide, and derivatives of thiosemicarbazide and dicarboximide were obtained. Next, cyclization reaction of dicarboximide derivatives in alkaline media was used to prepare 1,2,4-triazole-5-thione. The 4-oxo-1,3-thiazolidine was synthesized by the reaction of dicarboximide with ethyl bromoacetate. All obtained derivatives were analysed by ^{1}H and ^{13}C NMR spectra, and for one compound, the X-ray crystallography was done. Antimicrobial, antiviral and in vitro evaluations of cytotoxicity were examined. According to the preliminary antiviral screening, compounds **3** and **4** presented the antiviral activity against HSV-1 and CVB3. Additionally, compound **3** shows selective in vitro toxic effect against human epithelial cells FaDu, without affecting normal animal cell line (Vero). The same derivatives **3** and **4** also displayed a wide spectrum of antimicrobial activity against reference microorganisms and indicated both antibacterial and antifungal potential activities.

Keywords Thiosemicarbazide derivatives · Dicarboximide derivatives · 1,2,4-Triazole derivatives · 4-Oxo-1,3-thiazolidine derivatives · Antimicrobial activity · Antiviral activity

Introduction

Herpes simplex virus type 1 (HSV-1) is an enveloped dsDNA virus belonging to the Herpesviridae family [1]. It is regarded as one of the most common human viruses, affecting up to a 90% of worldwide population. Acquisition of HSV-1 results in a lifelong latent infection of sensory ganglion [2]. HSV-1 can cause epithelial lesions, especially orolabial and genital infections, as well as keratitis, encephalitis and neonatal infections [1, 2].

Mechanisms regulating the latency are not fully understood, but it is suggested that they involve interactions between immune and nervous system, infected cell signal transduction, infected cell transcriptional regulation and influence of the host's environment, e.g. stressful situations. HSV-1 latency can also be modulated by the endocrine system [3]. The role of thyroid hormone (T3) in regulation of herpesvirus replication during reactivation is believed to be the most significant. It may be due to the fact that T3 influences the activity of immune system, nervous system, cell signal transduction and transcriptional regulation. Thyroid hormone binds to the nuclear receptor and regulates cell proliferation, differentiation and apoptosis. Moreover, stress may cause fluctuations of T3 [4]. It was also shown that differentiated LNCaP cells (human neuron-like cells) resist HHV-1 replication upon stimulation with T3, probably through active PI3 K (phosphoinositide 3-kinase) signalling. The treatment with LY294002

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Published online: 15 January 2018

(2-morpholin-4-yl-8-phenylchromen-4-one), a PI3 K inhibitor, can reverse T3-mediated repression of viral replication in LNCaP cells [3].

Most of the drugs used for the management of herpesvirus infections target the DNA polymerase (Pol). Acyclovir and penciclovir, with their respective prodrugs valacyclovir and famciclovir, as well as foscarnet, and cidofovir are the most commonly used drugs [1]. However, the treatment, especially in case of immunosuppressed individuals, often leads to development of resistance caused by mutations in the viral thymidine kinase and/or DNA polymerase [1, 5].

Coxsackie B viruses (CVBs) are single-stranded, positive-sense RNA viruses belonging to the Picornaviridae family. CVBs may be responsible for a variety of diseases, from mild syndromes to life-threatening conditions such as pancreatitis, myocarditis, meningitis and encephalitis. Among newborns and young infants, the infections are significantly more severe and often lead to long-term sequelae [6, 7]. CVB3 is believed to be the most common cause of virus-induced myocarditis that may develop into dilated cardiomyopathy, which is the leading cause of heart failure among young adults [8].

There is no specific antiviral therapy for CVB3 infections, and the only possible treatment is symptomatic. In the past few decades, there had been many reports describing the molecules that block CVB replication in vitro, influencing different steps of the viral life-cycle. However, none of them proved to be effective and safe enough to be introduced into practical use. Pleconaril which is an inhibitor of viral RNA uncoating was the most promising drug candidate; however, due to the considerable side effects, low efficiency and emerging resistance it has not been approved by the FDA [6].

Due to the emergence of drug-resistant mutants of HSV-1 and lack of specific antiviral therapy for CVB3-induced myocarditis, it is crucial to look for new antiviral compounds.

Five-membered heterocyclic compounds especially 1,2,4-triazole, thiazolidin-4-one and their open-chain counterpart thiosemicarbazide and dicarboximide display a broad spectrum of biological activities [9–18] including antiviral [19–26] and antimicrobial [27–29] properties. What is important, derivatives based on this pharmacophore are already in the market and they have applications as clinically useful medicines. Among them are thiazolidomycin activity against Streptomyces species [30], ribavirin—antiviral [31], fluconazole—antifungal [31], itraconazole—antifungal [31], voriconazole—antifungal [31], ravuconazole—antifungal [31], terconazole—antifungal [32], albaconazole—antifungal [33], isavuconazole—antifungal [33] and ritonavir—antiretroviral [34].

In 2017 year, C.C. Pacca and colleagues described series of thiosemicarbazones and phthalyl-thiazoles with a potential antiviral activity against yellow fever virus (YFV) and Saint Louis encephalitis virus (SLEV). Based on the biological study, the authors stated that thiosemicarbazone derivatives presented antiviral activity against both YFV and SLEV, while phthalyl-thiazole derivatives were effective only against SLEV [35].

Our team has been working for many years on the synthesis of derivatives of heterocyclic compounds with the potential biological activity [36–40].

Recently, we have presented results concerning the synthesis and antimicrobial and antituberculosis evaluation of *N*-(substituted-thioureido)aminobicyclo dicarboximide and 3,4-disubstituted 1,2,4-triazolino-5-thione. It is worth noting that some of these compounds exhibited strong bioactivity. They were the most active against clinical isolates of *C. albicans*. Among them were N^4-phenyl-N^1-(2-carboxy-1,2,3,6-tetrahydrobenzoyl)thiosemicarbazide, *N*-(phenyl-thioureido)-aminobicyclo(1,2,3,6-tetrahydrophthaloyl)-dicarboximide and *N*-(phenyl-thioureido)-aminobicyclo[2.2.1]hept-5-ene dicarboximide with MIC = 15.6 µg/mL [41].

In the light of the above-mentioned literature and as an extension of our previous studies on synthesis and biological screening [39–43], we designed new analogues of thiosemicarbazide, dicarboximide and 1,2,4-triazole-5-thione with modification of their structure. Additionally, we designed and synthesized 1,2,3,6-tetrahydrophthaloyl-4-oxo-1,3-thiazolidine. Then, potential antimicrobial and antiviral activity of the synthesized compounds were explored.

Experimental

Materials and methods

Melting points were determined in a Fischer–Johns block and presented without any corrections. Elemental analysis was carried out using a PerkinElmer 2400 CHN Analyser. All results were in good agreement with calculated values. The error range was ± 0.4% for each element analysed. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Avance 300 MHz spectrometer in DMSO-d_6 and with TMS as an internal standard. Chemicals were purchased from Sigma-Aldrich (Munich, Germany) or Merck Co. (Darmstadt, Germany) and used without further purification.

Synthesis and characterization of thiosemicarbazide derivatives (1–4)

1.52 g (10 mmol) of 1,2,3,6-tetrahydrophtalic anhydride and 10 mmol of appropriate 4-substituted-3-thiosemicarbazide in dry chloroform were refluxed for 1 h. After cooling, the precipitate was filtered off and crystallized from ethanol–water.

N^4-ethyl-N^1-(2-carboxy-1,2,3,6-tetrahydrobenzoyl)thiosemicarbazide (**1**)

CAS Registry Number 1625646-62-9. Yield: 65%; M.p.: 158–159 °C. ^{1}H NMR (DMSO-d_6) δ (ppm) = 1.05 (t, 3H, J = 7.5 Hz, CH_3), 2.26 (s, 2H, CH_2), 2.49 (s, 2H, CH_2), 2.74 (s, 1H, CH), 3.02 (s, 1H, CH), 3.33–3.46 (m, 2H, CH_2), 5.62 (s, 2H, CH=CH), 7.38 (s, 1H, NH), 9.15 (s, 1H, NH), 9.71 (s, 1H, NH), 12.48 (s, 1H, COOH); ^{13}C NMR (DMSO-d_6) δ (ppm) = 17.70 (CH_3), 25.38 (CH_2), 26.70 (CH_2), 38.22 (CH-CONH), 38.93 (CH_2CH_3), 39.86 (CH-COOH), 123.33 (CH=CH), 123.91 (CH=CH), 171.09 (CONH), 174.09 (COOH), 179.91 (C=S).

N^4-phenyl-N^1-(2-carboxy-1,2,3,6-tetrahydrobenzoyl)thiosemicarbazide (**2**)

CAS Registry Number 1625646-63-0. Yield: 86%; M.p.: 143–145 °C. ^{1}H NMR (DMSO-d_6) δ (ppm) = 2.31 (s, 2H, CH_2), 2.49 (s, 2H, CH_2), 2.84 (s, 1H, CH), 3.09 (s, 1H, CH), 5.65 (s, 2H, CH=CH), 7.12–7.17 (m, 1H, *p*-Ph), 7.29–7.35 (m, 2H, *m*-Ph), 7.53–7.56 (m, 2H, *o*-Ph), 9.06 (s, 1H, NH), 9.64 (s, 1H, NH), 9.98 (s, 1H, NH), 12.53 (s, 1H, COOH); ^{13}C NMR (DMSO-d_6) δ (ppm) = 22.83 (CH_2), 26.24 (CH_2), 37.82 (CH-CONH), 39.77 (CH-COOH), 124.58 (*p*-PhCH), 124.86 (*o*-Ph2CH), 125.31 (CH=CH), 128.11 (*m*-Ph2CH), 138.92 (*i*-PhC), 172.67 (CONH), 175.80 (COOH), 180.50 (C=S).

N^4-(2-methylphenyl)-N^1-(2-carboxy-1,2,3,6-tetrahydrobenzoyl)thiosemicarbazide (**3**)

Yield: 60%; M.p.: 100–102 °C. ^{1}H NMR (DMSO-d_6) δ (ppm) = 2.14–2.41 (m, 7H, CH_3 + $2CH_2$), 3.24 (s, 2H, 2CH), 5.88 (s, 2H, CH=CH), 7.09–7.27 (m, 4H, Ph), 7.55 (s, 1H, NH), 9.08 (s, 1H, NH), 9.69 (s, 1H, NH), 10.07 (s, 1H, COOH); ^{13}C NMR (DMSO-d_6) δ (ppm) = 17.90 (CH_3), 23.38 (CH_2), 26.16 (CH_2), 37.59 (CH-CONH), 39.16 (CH-COOH), 125.94 (*o*-PhCH), 127.75 (*p*-PhCH), 128.20 (*m*-PhCH), 129.26 (CH=CH), 130.83 (*m*-PhCH), 133.72 (*o*-PhC-CH_3), 138.42 (*i*-PhC), 175.04 (CONH), 177.47 (COOH), 180.67 (C=S).

N^4-(4-chlorophenyl)-N^1-(2-carboxy-1,2,3,6-tetrahydrobenzoyl)thiosemicarbazide (**4**)

Yield: 62%; M.p.: 162–164 °C. ^{1}H NMR (DMSO-d_6) δ (ppm) = 2.33 (s, 2H, CH_2), 2.48 (s, 2H, CH_2), 2.87 (s, 1H, CH), 3.14 (s, 1H, CH), 5.68 (s, 2H, CH=CH), 7.39–7.42 (d, 2H, J = 8.6 Hz, *p*-Ph), 7.60–7.63 (d, 2H, J = 8.6 Hz, *p*-Ph), 9.16 (s, 1H, NH), 9.81 (s, 1H, NH), 10.04 (s, 1H, NH), 12.21 (s, 1H, COOH); ^{13}C NMR (DMSO-d_6) δ (ppm) = 25.34 (CH_2), 26.75 (CH_2), 38.33 (CH-CONH), 125.79 (*o*-Ph2CH), 126.76 (CH=CH), 128.5 (*m*-Ph2CH), 129.36 (*p*-PhC-Cl), 138.87 (*i*-PhC), 173.19 (CONH), 176.41 (COOH), 180.90 (C=S).

Synthesis and characterization of *N*-(substituted-thioureido)aminobicyclo(1,2,3,6-tetrahydrophthaloyl) dicarboximide (5–7)

The thiosemicarbazide **1–3** (2 mmol) was dissolved in a mixture of glacial acetic acid (4 mL) and concentrated sulphuric acid (2 mL). The solution was left at room temperature for 1 h, then poured into crushed ice and kept for 2 h at room temperature. The precipitated solid was filtered off and crystallized from ethanol.

N-(ethyl-thioureido)aminobicyclo(1,2,3,6-tetrahydrophthaloyl) dicarboximide (**5**)

CAS Registry Number 1625646-65-2. Yield: 62%; M.p. 164–166 °C. ^{1}H NMR (DMSO-d_6) δ (ppm) = 1.11 (t, 3H, J = 7.5 Hz, CH_3), 2.29–2.50 (m, 4H, $2CH_2$), 2.58 (s, 1H, CH), 3.41 (s, 1H, CH), 3.46–3.51 (m, 2H, CH_2), 5.93 (s, 2H, CH=CH), 8.28 (s, 1H, NH), 9.70 (s, 1H, NH); ^{13}C NMR (DMSO-d_6) δ (ppm) = 14.29 (CH_3), 23.12 ($2CH_2$), 37.20 (CH_2CH_3), 38.90 (CH-CO), 39.90 (CH-CO), 127.68 (CH=CH), 177.32 (2C=O), 181.38 (C=S).

N-(phenyl-thioureido)aminobicyclo(1,2,3,6-tetrahydrophthaloyl) dicarboximide (**6**)

CAS Registry Number 1625646-67-4. Yield: 82%; M.p. 164–166 °C. ^{1}H NMR (DMSO-d_6) δ (ppm) = 2.30–2.39 (m, 4H, $2CH_2$), 3.40 (s, 2H, 2CH), 5.89 (s, 2H, CH=CH), 7.07–7.22 (m, 1H, *p*-Ph), 7.30–7.58 (m, 4H, *o*-Ph + *m*-Ph), 10.05 (s, 2H, 2NH); ^{13}C NMR (DMSO-d_6) δ (ppm) = 22.80 ($2CH_2$), 36.89 (2CH-CO), 124.78 (*p*-PhCH), 127.37 (*o*-Ph2CH), 128.48 (*m*-Ph2CH + CH=CH), 138.51 (*i*-PhC), 176.79 (2C=O), 180.93 (C=S).

N-(2-methylphenyl-thioureido)aminobicyclo(1,2,3,6-tetrahydrophthaloyl) dicarboximide (**7**)

Yield: 51%; M.p. 110–112 °C. ^{1}H NMR (DMSO-d_6) δ (ppm) = 2.14–2.41 (m, 7H, CH_3 + $2CH_2$), 3.24 (s, 2H, 2CH), 5.89 (s, 2H, CH=CH), 7.08–7.27 (m, 4H, Ph), 9.69 (bs, 1H, NH), 10.07 (bs, 1H, NH); ^{13}C NMR (DMSO-d_6) δ (ppm) = 17.90 (CH_3), 23.37 ($2CH_2$), 37.54 (2CH-CO), 126.69 (*o*-PhCH), 127.74 (*p*-PhCH), 127.99 (*m*-PhCH), 129.31 (CH=CH), 130.82 (*m*-PhCH), 136.34 (*o*-PhC-CH_3), 137.50 (*i*-PhC), 177.46 (2C=O), 181.71 (C=S).

N-(4-chlorophenyl-thioureido)aminobicyclo(1,2,3,6-tetrahydrophthaloyl) dicarboximide (8)

Yield: 62%; M.p.: 169–171 °C. ^{1}H NMR (DMSO-d_6) δ (ppm) = 2.26–2.30 (m, 4H, $2CH_2$), 2.87 (s, 1H, CH), 3.26 (s, 2H, 2CH), 5.89 (s, 2H, CH=CH), 7.34–7.43 (m, 2H, *p*-Ph), 7.58–7.61 (m, 2H, *p*-Ph), 10.06 (s, 1H, NH), 10.19 (bs, 1H, NH); ^{13}C NMR (DMSO-d_6) δ (ppm) = 23.34 ($2CH_2$), 37.42 (2CH-CO), 118.81 (*o*-Ph2CH), 124.90 (*p*-PhC-Cl), 127.97 (CH=CH), 129.27 (*m*-Ph2CH), 140.42 (*i*-PhC), 177.27 (2C=O), 181.90 (C=S).

Synthesis and characterization of bis *N*-(substituted-thioureido)aminobicyclo[2.2.2] oct-7-ene-2,3,5,6-tetracarboximide (9–10)

10 mmol of bicyclo[2.2.2]oct-7-ene-2,3,5,6-tetracarboxylic dianhydride and 20 mmol of appropriate 4-substituted-3-thiosemicarbazide in dry chloroform were refluxed for 1 h. After cooling, the precipitate was filtered off and crystallized from acetonitrile + methanol (1:1).

Bis *N*-(ethyl-thioureido)aminobicyclo[2.2.2]-oct-7-ene-2,3,5,6-tetracarboximide (9)

Yield: 63%; M.p. 125–127 °C. ^{1}H NMR (DMSO-d_6) δ (ppm) = 1.03 (t, 6H, J = 7.0 Hz, $2CH_3$), 3.36–3.3.41 (m, 10H, 6CH + $2CH_2$), 6.01 (s, 2H, CH=CH), 8.24 (bs, 2H, 2NH), 9.75 (bs, 2H, 2NH); ^{13}C NMR (DMSO-d_6) δ (ppm) = 14.57 (CH_3), 33.69 (2CH), 39.31 (CH_2-CH_3), 41.49 (4CH-CO), 131.32 (CH=CH), 173.73 (4C=O), 181.50 (2C=S).

Bis *N*-(phenyl-thioureido)aminobicyclo[2.2.2]-oct-7-ene-2,3,5,6-tetracarboximide (10)

CAS Registry Number 1625646-83-4. Yield: 83%; M.p. 220–222 °C. ^{1}H NMR (DMSO-d_6) δ (ppm) = 3.33 (s, 4H, 4CH), 3.47 (s, 2H, 2CH) 6.38 (s, 2H, CH=CH), 7.08–7.36 (m, 4H, *m*-Ph), 7.44–7.62 (m, 6H, *o*-Ph + *p*-Ph), 9.92 (bs, 2H, 2NH), 10.18 (s, 2H, 2NH); ^{13}C NMR (DMSO-d_6) δ (ppm) = 33.23 (2CH), 40.33 (4CH-CO), 125.62 (*p*-Ph2CH), 128.45 (*o*-Ph4CH), 130.90 (*m*-Ph4CH + CH=CH), 138.43 (*i*-Ph2C), 174.18 (4C=O), 180.69 (2C=S).

Synthesis and characterization of 3,4-disubstituted 1,2,4-triazolino-5-thione (11–15)

Method A (11–15)

A solution of *N*-(substituted-thioureido)aminobicyclo dicarboximide **5–9** (2 mmol) in 2% sodium hydroxide (5 mL) was refluxed for 2 h. After cooling, the reaction mixture was filtered off and isolated solution was acidified with 3 M HCl. The precipitate was filtered off and crystallized from ethanol.

Method B (11–13)

A solution of N^4-substituted-N^1-(2-carboxy-1,2,3,6-tetrahydrobenzoyl)thiosemicarbazide **1–3** (2 mmol) in 2% sodium hydroxide (5 mL) was refluxed for 2 h. After cooling, the reaction mixture was filtered off and isolated solution was acidified with 3 M HCl. The precipitate was filtered off and crystallized from ethanol.

1-(4-Ethyl-1,2,4-triazol-5-thione-3-yl)-cyclohex-4-ene-2-carboxylic acid (11)

CAS Registry Number 1625646-73-2. Yield: 58%; M.p. 204–206 °C. ^{1}H NMR (DMSO-d_6) δ (ppm) = 1.30 (t, 3H, J = 7.5 Hz, CH_3), 3.02 (s, 1H, CH), 3.39 (s, 4H, $2CH_2$), 3.62 (s, 1H, CH), 4.07 (q, 2H, J = 7.5 Hz, CH_2CH_3), 3.44 (s, 2H, 2CH), 5.60 (d, 1H, J = 10.0 Hz, CH=CH), 5.79 (d, 1H, J = 10.0 Hz, CH=CH), 12.36 (bs, 1H, NH), 13.49 (s, 1H, COOH); ^{13}C NMR (DMSO-d_6) δ (ppm) = 13.81 (CH_3), 25.42 (CH_2), 28.12 (CH_2), 29.85 (CH-triazole) 38.52 (CH_2CH_3), 39.95 (CH-COOH) 123.46 (CH=CH), 126.54 (CH=CH), 153.64 ($C_{triazole}$), 166.16 (COOH), 174.55 (C=S).

1-(4-Phenyl-1,2,4-triazol-5-thione-3-yl)-cyclohex-4-ene-2-carboxylic acid (12)

Yield: 78%; M.p. 271–273 °C. ^{1}H NMR (DMSO-d_6) δ (ppm) = 2.16–2.18 (m, 4H, $2CH_2$), 2.68–2.74 (m, 1H, CH), 3.19 (q, 1H, J = 5.2 Hz, CH), 5.52 (d, 1H, J = 10.1 Hz, CH=CH), 5.70 (d, 1H, J = 9.8 Hz, CH=CH), 7.37–7.39 (m, 2H, *m*-Ph), 7.52–7.63 (m, 3H, *o*-Ph + *p*-Ph), 12.31 (bs, 1H, NH), 13.65 (s, 1H, COOH); ^{13}C NMR (DMSO-d_6) δ (ppm) = 25.13 (CH_2), 26.61 (CH_2), 29.97 (CH-triazole), 38.93 (CH-COOH) 123.22 (CH=CH), 125.91 (CH=CH), 128.47 (*o*-Ph2CH), 129.60 (*m*-Ph2CH + *p*-PhCH), 133.95 (*i*-PhC), 153.38 ($C_{triazole}$), 167.37 (COOH), 174.05 (C=S).

1-[4-(2-Methylphenyl)-1,2,4-triazol-5-thione-3-yl]-cyclohex-4-ene-2-carboxylic acid (13)

Yield: 55%; M.p. 242–244 °C. ^{1}H NMR (DMSO-d_6) δ (ppm) = 1.96–2.38 (m, 7H, CH_3 + $2CH_2$), 2.55–3.15 (m, 2H, 2CH), 5.52 (d, 1H, J = 9.0 Hz, CH=CH), 5.69 (d, 1H, J = 9.0 Hz, CH=CH), 7.19–7.49 (m, 4H, Ph), 12.25 (bs, 1H, NH), 13.68 (s, 1H, COOH); ^{13}C NMR (DMSO-d_6) δ (ppm) = 17.96 (CH_3), 25.67 (CH_2), 27.45 (CH_2), 30.79 (CH-triazole), 39.43 (CH-COOH), 126.61 (*o*-PhCH), 127.77 (*p*-PhCH), 128.89 (*m*-PhCH), 129.41 (CH=CH), 130.49

(*m*-PhCH), 136.81 (*o*-PhC-CH_3), 137.33 (*i*-PhC), 153.65 ($C_{triazole}$), 167.26 (COOH), 174.64 (C=S).

2,6-[Bis-(4-ethyl-1,2,4-triazol-5-thione-3-yl)]-bicyclo[2.2.2]oct-7-ene-3,5-dicarboxylic acid (14)

Yield: 51%; M.p. 262–264 °C. ^{1}H NMR (DMSO-d_6) δ (ppm) = 1.24 (t, 6H, J = 7.5 Hz, $2CH_3$), 3.05–3.35 (m, 4H, 4CH), 3.56–3.67 (m, 2H, 2CH), 3.88–3.99 (m, 4H, $2CH_2$), 6.22 (s, 2H, CH=CH), 10.72 (bs, 2H, 2NH), 12.66 (s, 2H, 2COOH); ^{13}C NMR (DMSO-d_6) δ (ppm) = 13.89 ($2CH_3$), 36.76 (2CH), 37.57 (2CH-triazole), 38.21 ($2CH_2$-CH_3), 50.42 (2CH-COOH), 131.59 (CH=CH), 154.75 ($2C_{triazole}$), 165.24 (2COOH), 174.02 (2C=S).

2,6-[Bis-(4-phenyl-1,2,4-triazol-5-thione-3-yl)]-bicyclo[2.2.2]oct-7-ene-3,5-dicarboxylic acid (15)

CAS Registry Number 1625646-85-6. Yield: 57%; M.p. 287–289 °C. ^{1}H NMR (DMSO-d_6) δ (ppm) = 2.71–3.24 (m, 6H, 6CH), 6.21–6.30 (m, 1H, CH=CH), 6.36–6.41 (m, 1H, CH=CH), 7.28–7.30 (m, 4H, *m*-Ph), 7.45–7.59 (m, 6H, *o*-Ph + *p*-Ph), 12.08 (bs, 2H, 2NH), 13.50 (s, 1H, COOH), 13.54 (s, 1H, COOH); ^{13}C NMR (DMSO-d_6) δ (ppm) = 34.59 (2CH), 37.75 (2CH-triazole), 47.29 (2CH-COOH), 128.76 (*p*-Ph2CH), 129.52 (*o*-Ph4CH), 131.47 (*m*-Ph4CH + CH=CH), 133.76 (*i*-Ph2C), 152.90 ($2C_{triazole}$), 166.90 (2COOH), 174.34 (2C=S).

Synthesis and characterization of *N*-[(4-oxo-3-substituted-1,3-thiazolidin-2-ylimine)]-bicyclo(2,3,5,6-tetrahydrophthaloyl) dicarboximide (16–20)

To a suspension of *N*-(substituted-thioureido)aminobicyclo dicarboximide (2.5 mmol) in absolute ethanol (25 mL), anhydrous sodium acetate (10 mmol) and ethyl bromoacetate (2.5 mmol) were added and mixture was refluxed for 4 h. After cooling, the solution was allowed to stand overnight. The precipitate was filtered off and crystallized from ethanol.

N-[(4-oxo-3-ethyl-1,3-thiazolidin-2-ylimine)]-bicyclo(1,2,3,6-tetrahydrophthaloyl) dicarboximide (16)

Yield: 76%; M.p. 162–164 °C. ^{1}H NMR (DMSO-d_6) δ (ppm) = 1.15 (t, 3H, J = 6.0 Hz, CH_3), 2.17–2.25 (m, 2H, CH_2), 2.39–2.45 (m, 2H, CH_2), 3.24–3.26 (m, 2H, 2CH), 3.74 (q, 2H, J = 6.0, 15.0 Hz CH_2CH_3), 4.10 (s, 2H, CH_2), 5.92–5.94 (m, 2H, CH=CH). ^{13}C NMR (DMSO-d_6) δ (ppm) = 12.29 (CH_3), 23.82 ($2CH_2$), 33.42 ($CH_{2thiazole}$), 38.19 (CH_2-CH_3), 38.33 (2CH-CO), 128.36 (CH=CH), 169.58 ($C_{thiazole}$), 172.10 (C=O), 175.40 (2C=O).

N-[(4-oxo-3-phenyl-1,3-thiazolidin-2-ylimine)]-bicyclo(1,2,3,6-tetrahydrophthaloyl) dicarboximide (17)

Yield: 85%; M.p. 232–234 °C. ^{1}H NMR (DMSO-d_6) δ (ppm) = 2.16–2.25 (m, 2H, CH_2), 2.39–2.52 (m, 2H, CH_2), 3.21 (s, 2H, 2CH), 4.34 (s, 2H, CH_2), 5.95 (s, 2H, CH=CH), 7.24–7.58 (m, 5H, Ph). ^{13}C NMR (DMSO-d_6) δ (ppm) = 23.82 ($2CH_2$), 33.65 ($CH_{2thiazole}$), 38.17 (2CH-CO), 128.35 (CH=CH), 128.49 (*o*-Ph2CH), 129.38 (*p*-PhCH), 129.63 (*m*-Ph2CH), 134.72 (*i*-PhC), 170.72 ($C_{thiazole}$), 171.99 (C=O), 179.26 (2C=O).

N-[(4-oxo-3-(2-methylphenyl)-1,3-thiazolidin-2-ylimine)]-bicyclo(1,2,3,6-tetrahydrophthaloyl) dicarboximide (18)

Yield: 72%; M.p. 250–252 °C. ^{1}H NMR (DMSO-d_6) δ (ppm) = 2.20–2.41 (m, 5H, CH_3 + CH_2), 2.49 (s, 1H, CH), 3.19 (s, 2H, CH_2), 3.31 (s, 1H, CH), 4.29 (s, 2H, CH_2), 5.91 (s, 2H, CH=CH), 7.25–7.36 (m, 4H, Ph). ^{13}C NMR (DMSO-d_6) δ (ppm) = 17.42 (CH_3), 23.82 ($2CH_2$), 33.65 ($CH_{2thiazole}$), 38.18 (2CH-CO), 127.44 (*o*-PhCH), 128.35 (CH=CH), 129.04 (*p*-PhCH), 130.00 (*m*-PhCH), 131.29 (*m*-PhCH), 133.98 (*o*-PhC-CH_3), 136.45 (*i*-PhC), 169.78 ($C_{thiazole}$), 171.88 (C=O), 175.27 (2C=O).

Bis *N*-[(4-oxo-3-ethyl-1,3-thiazolidin-2-ylimine)]-bicyclo[2.2.2]-oct-7-ene-2,3,5,6-tetracarboximide (19)

Yield: 77%; M.p. 316–318 °C. ^{1}H NMR (DMSO-d_6) δ (ppm) = 1.16 (t, 6H, J = 7.0 Hz, $2CH_3$), 3.34 (s, 4H, 4CH), 3.46 (s, 2H, 2CH), 3.72 (q, 4H, J = 7.0, 14.0 Hz $2CH_2CH_3$), 4.12 (s, 4H, $2CH_2$), 6.27 (d, 1H, J = 3.0 Hz, CH=CH), 6.28 (d, 1H, J = 3.0 Hz, CH=CH),). ^{13}C NMR (DMSO-d_6) δ (ppm) = 12.31 ($2CH_3$), 33.60 ($2CH_{2thiazole}$), 34.37 (2CH), 38.37 ($2CH_2CH_3$), 41.93 (4CH-CO), 131.95 (CH=CH), 169.77 ($2C_{thiazole}$), 172.09 (2C=O), 172.73 (4C=O).

Bis *N*-[(4-oxo-3-phenyl-1,3-thiazolidin-2-ylimine)]-bicyclo[2.2.2]-oct-7-ene-2,3,5,6-tetracarboximide (20)

Yield: 80%; M.p. 318–320 °C. ^{1}H NMR (DMSO-d_6) δ (ppm) = 3.29 (s, 6H, 6CH), 4.26 (s, 4H, $2CH_2$), 6.24 (d, 1H, J = 3.0 Hz, CH=CH), 6.27 (d, 1H, J = 3.0 Hz, CH=CH), 7.34–7.39 (m, 4H, *m*-Ph), 7.46–7.57 (m, 6H, *o*-Ph + *p*-Ph). ^{13}C NMR (DMSO-d_6) δ (ppm) = 33.76 ($2CH_{2thiazole}$), 34.34 (2CH), 41.86 (4CH-CO), 128.49 (*p*-Ph2CH), 129.38 (*o*-Ph4CH), 129.62 (*m*-Ph4CH), 131.89 (CH=CH), 134.75

(*i*-Ph2C), 170.82 ($2C_{thiazole}$), 171.89 (2C=O), 172.43 (4C=O).

X-ray crystallography

X-ray data of compound **17** were collected on the Kuma KM-4 diffractometer: crystal size 0.60 × 0.60 × 0.40 mm, MoKα (λ = 0.71073 Å) radiation, $\omega/2\theta$ scans, T = 293(2) K, absorption correction: Xabs2 [44], T_{min}/T_{max} of 0.4542/2.0561. The structure was solved by direct methods using SHELXS-2013/1 [45] and refined by full-matrix least-squares with SHELXL-2014/7 [45]. All H atoms were positioned geometrically, treated as riding on their parent C atoms with C–H distances of 0.98 Å (CH), 0.97 Å (CH_2) and 0.93 Å (aromatic) and refined with isotropic displacement parameters taken as 1.5 times of those of the respective parent atoms. All calculations were performed using WINGX version 2014.1 package [46]. CCDC-1556158 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge at www.ccdc.cam.ac.uk/conts/retrieving.html [or from the Cambridge Crystallographic Data Centre (CCDC), 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44(0) 1223 336 033; email: deposit@ccdc.cam.ac.uk].

Crystal data of ***17***: $C_{17}H_{15}N_3O_3S$, M = 341.38, triclinic, space group $P\bar{1}$, a = 9.959(2), b = 12.280(2)(3), c = 13.481(2) Å, α = 91.51(2), β = 96.55(2), γ = 103.50(2)°, V = 1590.3(5) Å^3, Z = 4, d_{calc} = 1.426 Mg m^{-3}, $F(000)$ = 712, μ(Mo Kα) = 0.225 mm^{-1}, T = 293(2) K, 9304 measured reflections (θ range 2.12–30.07), 9304 unique reflections, final R = 0.069, wR = 0.199, S = 1.085 for 5047 reflections with $I > 2\sigma(I)$.

Antiviral activity

Cells and viruses

The Vero cell culture (ECACC No. 84113001—established from a kidney of a normal adult African Green monkey) and the FaDu cell culture (ATCC HTB-43 established from a punch biopsy of an hypopharyngeal tumour removed from a Hindu patient) were used in the experiment. The media in the culture (Dulbecco's modified Eagle medium—DMEM and modified Eagle medium—MEM, Sigma-Aldrich, Saint Louis, MO, USA) were supplemented with 10% foetal bovine serum—FBS (Sigma-Aldrich, Saint Louis, MO, USA), 100 U/mL of penicillin and 0.1 mg/mL of streptomycin (Polfa Tarchomin, Warsaw, Poland). The cell culture was incubated at 37 °C in the 5% CO_2 atmosphere.

For antiviral activity of the examined compounds, the Herpes simplex virus type l—HSV-1 (ATCC No. VR-260) and coxsackievirus B3—CVB3 (ATCC No. VR-30) from the American Type Culture Collection were used. The viruses were propagated in the Vero cell culture. Virus stocks were stored at − 70 °C until used.

Cytotoxicity assay

Compounds were dissolved in dimethyl sulphoxide—DMSO (POCH, Gliwice, Poland) in the concentration of 50 mg/mL and further diluted with a complete test medium. 100 µL of the cell culture prepared was seeded into 96-well plastic plates (Becton Dickinson and Company, Franklin Lakes, NJ USA) with a cell density of 1.5 × 10^4 (Vero) and 2 × 10^4 (FaDu) cells per well. After a 24 h incubation at 37 °C, the media were removed and the cells were treated with a solution of the examined substance diluted in the media with 2% of the serum. The cells were submitted to a series of compound concentrations, from 1000 to 1.9 µg/mL. Twofold serial dilutions of compounds were added to the cells in triplicates. The cell cultures were incubated for 72 h at 37 °C in the 5% CO_2 atmosphere.

Cytotoxicity of the tested compounds was estimated with the use of the MTT method and described by Takenouchi and Munekata, with modifications [47, 48]. The MTT method is a quantitative colorimetric toxicity test, based on the transformation of yellow, soluble tetrazolium salts (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) to purple–blue insoluble formazan. This process occurs naturally in mitochondria of living cells. After a 72-h incubation with compounds, cell cultures were supplemented with 10 µL per well of 5 mg/mL MTT (Sigma-Aldrich, Saint Louis, MO, USA) stock in PBS (BIOMED, Lublin, Poland) and the incubation was continued for 4 h at 37 °C. Then, 100 µL of aqueous solution containing 50% dimethylformamide (POCH, Gliwice, Poland) and 20% SDS (Sigma-Aldrich, Saint Louis, MO, USA) was added to solubilize the insoluble formazan precipitates produced by MTT. After the all-night incubation, the absorbance was measured by the Epoch plate reader (BioTek, Winooski, Vermont, USA) at two wavelengths—540 and 620 nm. On the basis of the test results, the cytotoxic concentration (CC_{50}), which is the amount of tested substance that is required to reduce the number of viable cells by 50% compared to the control culture, was determined and calculated using the Gen 5 2.01.14 software (BioTek, Winooski, Vermont, USA). Cell viability (%) was calculated as ($A_{540/620}$ of the treated/$A_{540/620}$ of the control) × 100. When the CC_{50} value was calculated, nontoxic concentrations of the examined compounds were selected to assess their antiviral activity. The investigation was carried out in triplicate.

Antiviral activity assay

Screening of antiviral activity by cytopathic effect reduction assay

The Vero cells, after a 24 h incubation, were infected with viruses in the dose of 100 $TCID_{50}$/mL. After 1 h incubation at 37 °C, the suspension of the viruses was removed and the media with 2% of serum together with the tested compounds in the nontoxic concentration were added to the cell cultures. The viruses diluted in the culture media without the tested compounds were used as a control. Acyclovir and ribavirin (Sigma-Aldrich, Saint Louis, MO, USA) were used as reference compounds. After a 48–72 h incubation at 37 °C, the cytopathic effect (CPE) of the viruses was examined by a light microscope. The antiviral activity of compounds was evaluated by direct observation of CPE reduction.

Determination of $TCID_{50}$ by the virus titre reduction method

After the evaluation of the antiviral activity of compounds, the cells were frozen, and after thawing, the viruses were titrated in 96-well plastic plates with the Vero cell culture. The titration of the viruses was performed using end-point titration technique. Vero cells were cultivated for 24 h at 37 °C in 96-well flat-bottomed plates. Growth medium was removed and tenfold dilutions of the virus in the absence of the samples (virus control) and compound-treated infected cells were added in quadruplicate and plates were incubated at 37 °C until typical cytopathic effect (CPE) was visible. The titres of the virus were estimated according to the Reed–Muench method, expressed as 50% tissue infectious dose ($TCID_{50}$) per mL [49].

In vitro antimicrobial assay

The examined compounds: **1–20** were screened in vitro for antibacterial and antifungal activities using the broth microdilution method according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) [50] and Clinical and Laboratory Standards Institute guidelines [51] against a panel of reference strains of microorganisms, including Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* ATCC 6538, *Staphylococcus epidermidis* ATCC 12228, *Bacillus subtilis* ATCC 6633, *Bacillus cereus* ATCC 10876, *Micrococcus luteus* ATCC 10240), Gram-negative bacteria (*Bordetella bronchiseptica* ATCC 4617, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13883, *Proteus mirabilis* ATCC 12453, *Salmonella typhimurium* ATCC 14028, *Pseudomonas aeruginosa* ATCC 9027) and fungi belonging to yeasts (*Candida albicans* ATCC 10231, *Candida parapsilosis* ATCC 22019). The microorganisms belonging to ATCC came from American Type Culture Collection, routinely used for the evaluation of antimicrobials. All the used microbial cultures were first subcultured on nutrient agar or Sabouraud agar for bacteria and fungi, respectively.

The surface of Mueller–Hinton agar (for bacteria) and RPMI 1640 with MOPS (for fungi) were inoculated with the suspensions of bacterial or fungal species. Microbial suspensions were prepared in sterile saline (0.85% NaCl) with an optical density of McFarland standard scale 0.5. Samples containing examined compounds were dissolved in 1 mL dimethyl sulphoxide (DMSO). Furthermore, bacterial and fungal suspensions were put onto Petri dishes with solid media containing 2000 µg/mL of the tested compounds followed incubation. The inhibition of microbial growth was judged by comparison with a control culture prepared without any sample tested. Ciprofloxacin or fluconazole (Sigma) were used as reference antibacterial or antifungal compounds, respectively.

Subsequently MIC (minimal inhibitory concentration) of the compounds was examined by the microdilution broth method, using their twofold dilutions in Mueller–Hinton broth (for bacteria) and RPMI 1640 broth with MOPS (for fungi) prepared in 96-well polystyrene plates. Final concentrations of the compounds ranged from 1000 to 0.488 µg/mL. Microbial suspensions were prepared in sterile saline (0.85% NaCl) with an optical density of 0.5 McFarland standard. Next bacterial or fungal suspension was added per each well containing 200 µL broth and various concentrations of the examined compounds. After incubation, the MIC was assessed spectrophotometrically as the lowest concentration of the samples showing complete bacterial or fungal growth inhibition. Appropriate DMSO, growth and sterile controls were carried out. The medium with no tested substances was used as control.

The MBC (minimal bactericidal concentration) or MFC (minimal fungicidal concentration) is defined as the lowest concentration of the compounds that is required to kill a particular bacterial or fungal species. MBC or MFC was determined by removing the culture used for MIC determinations from each well and spotting onto appropriate agar medium. The plates were incubated. The lowest compound concentrations with no visible growth observed was assessed as a bactericidal or fungicidal concentration. All the experiments were repeated three times and representative data are presented [43, 52, 53].

In this study, no bioactivity was defined as a MIC > 1000 µg/mL, mild bioactivity as a MIC in the range 501–1000 µg/mL, moderate bioactivity with MIC from 126 to 500 µg/mL, good bioactivity as a MIC in the range 26–125 µg/mL, strong bioactivity with MIC between 10 and 25 µg/mL and very strong bioactivity as a MIC < 10 µg/mL. The MBC/MIC or MFC/MIC ratios were calculated in order to determine bactericidal/fungicidal (MBC/MIC ≤ 4,

MFC/MIC ≤ 4) or bacteriostatic/fungistatic (MBC/MIC > 4, MFC/MIC > 4) effect of the tested compounds [52].

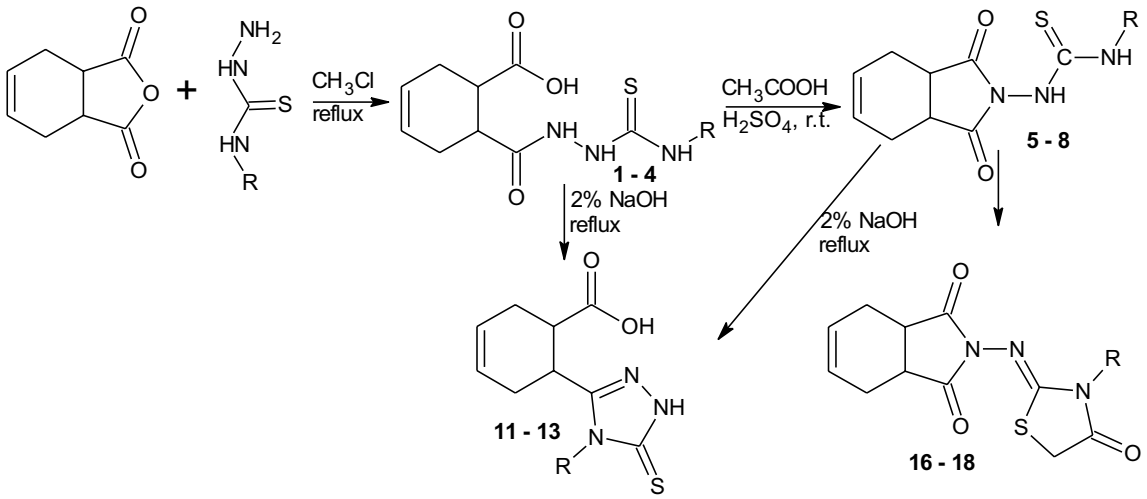
Results and discussion

Chemistry

The main goal of this investigation was synthesis, identification and in vitro antimicrobial and antiviral screening of thiosemicarbazide, di- and tetracarboximide, 1,2,4-triazole-5-thione and 4-oxo-1,3-thiazolidine. The synthetic pathway is presented in Schemes 1 and 2. Derivatives **3**, **4**, **7**, **8**, **9**, **13**, **14**, **16–20** are unknown in scientific literature.

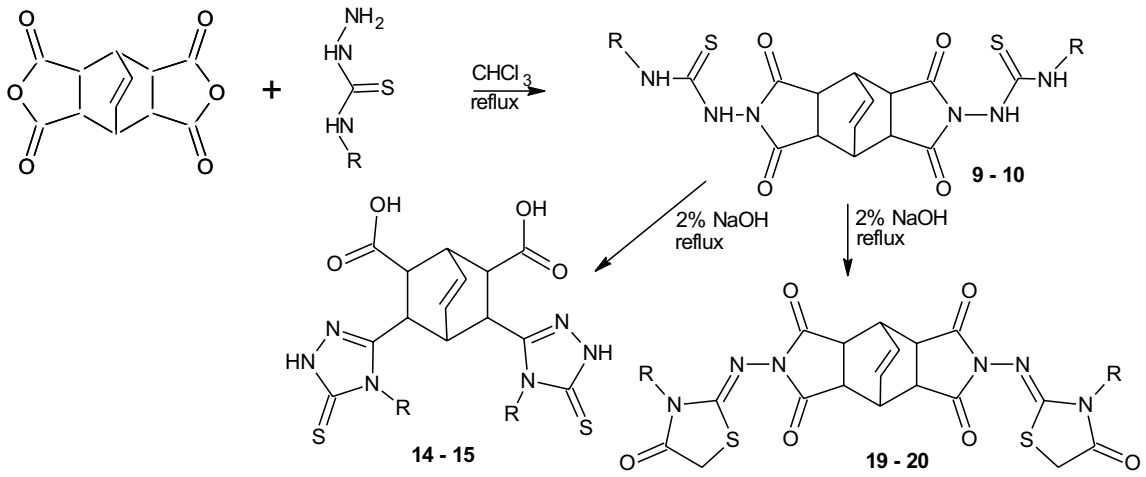
Selected dicarboxylic acid anhydrides were used as an initial compound. Linear derivatives of thiosemicarbazide (**1–4**) and tetracarboximide (**9–10**) were prepared by heating anhydride with 4-substituted-3-thiosemicarbazide in dry chloroform. Dicarboximides (**5–8**) were obtained in the cyclization reactions of linear thiosemicarbazides (**1–4**) in a mixture of concentrated sulphuric acid and glacial acetic acid. In the case of bicyclo[2.2.2]oct-7-ene-2,3,5,6-tetracarboxylic anhydride, the attempts to obtain the linear thiosemicarbazides failed and bis *N*-(substituted-thioureido) aminobicyclo tetracarboximides were prepared directly. Next new 3,4-disubstituted 1,2,4-triazolino-5-thione (**11–15**) were synthesized by the cyclization reaction of corresponding thiosemicarbazide, di- and tetracarboximide derivatives in alkaline media. Reaction of *N*-(substituted-thioureido) aminobicyclo di- and tetracarboximides with ethyl bromoacetate in the presence of sodium acetate in absolute ethanol led to the formation of 4-oxo-1,3-thiazolidine (**16–20**). This preparation is based on the most applied method—Hantzsch synthesis—in which α-halocarbonyl compounds react with thioamides [31, 54, 55]. Probably, sulphur atom of thioureido moiety attacked halogen atom of ethyl bromoacetate and then elimination of ethanol and hydrogen bromide took place and 1,3-thiazolidine was formed without selected intermediate (Fig. 1) [54]. Derivatives with thioureido group possess two positions for nucleophilic attacks, so two different structural isomers **A** and **B** can be obtained (Fig. 1) [56]. According to the crystal structure of **17**, it can be speculated that only one structural isomer **A** (Fig. 1) that is *N*-[(4-oxo-3-substituted-1,3-thiazolidin-2-ylimine)]-bicyclo(2,3,5,6-tetrahydrophthaloyl) dicarboximide has been synthesized. This is consistent with the description in the literature [57]. Unfortunately, in the case of N^4-(4-chlorophenyl)-N^1-(2-carboxy-1,2,3,6-tetrahydrobenzoyl)thiosemicarbazide, cyclization reaction failed and triazole and 1,3-thiazolidine were not obtained. The chemical structure of all obtained compounds was confirmed and proved by ^{1}H and ^{13}C NMR spectra.

The ^{1}H NMR spectra of compounds **1–4** showed three peaks due to the presence of the three NH groups



Compound No	R
1, 5, 11, 16	C_2H_5
2, 6, 12, 17	C_6H_5
3, 7, 13, 18	2-$CH_3C_6H_4$
4, 8	4-ClC_6H_4

Scheme 1 Synthesis of compounds **1–8**, **11–13** and **16–18**



Compound No	R
9, 14, 19	C_2H_5
10, 15, 20	C_6H_5

Scheme 2 Synthesis of compounds **9–10**, **14–15** and **19–20**

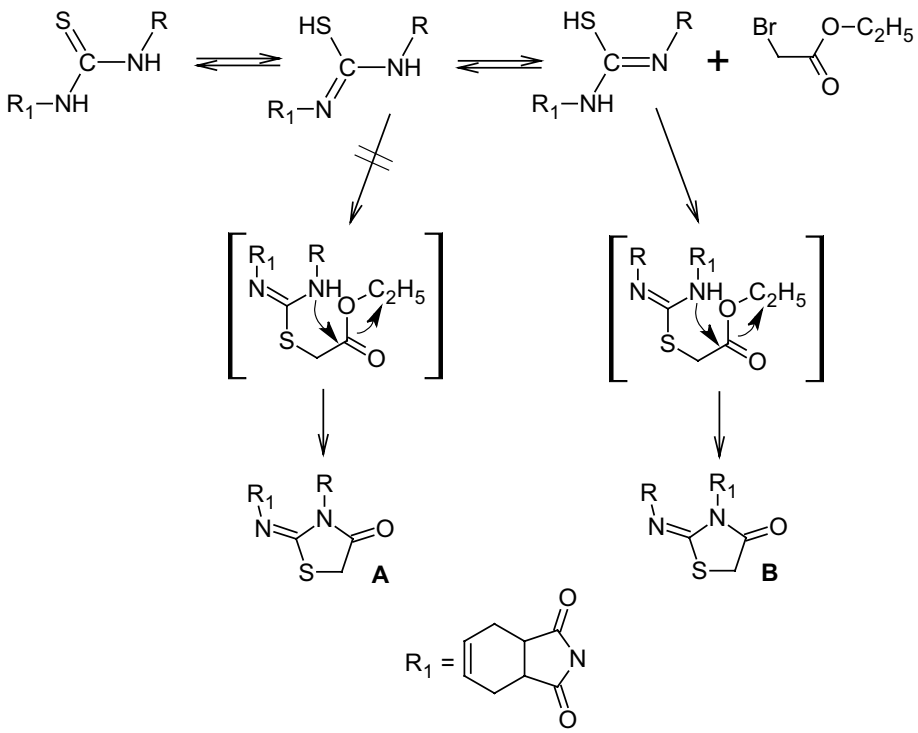


Fig. 1 Probably mechanism leading to the formation of 1,3-thiazolidine

between δ 7.38–10.04 ppm. In the ^{13}C NMR spectra of these compounds, corresponding two signals for C=O group and C=S group were displayed at 171.09–173.19 and 179.91–180.90 ppm, respectively, whereas ^{1}H NMR spectra for the carboximides (**5–10**) displayed one or two typical proton signals of the two NH groups in the region of 8.28–10.19 ppm. In the ^{13}C NMR spectra for compounds **5–10**, typical signals corresponding to C=O groups of cyclic carboximides and for C=S group of linear chain were observed. They resonated between 174.09–177.32 ppm and 180.69–181.38 ppm, respectively. ^{1}H NMR spectra for cyclic 1,2,4-triazole-5-thione **11–15** showed typical proton

signal of the NH group in the range of 10.72–12.36 ppm. In the ^{13}C NMR spectra for these compounds, carbon of triazole ring resonates in the range of 152.90–154.75 ppm, whereas carbon corresponds to C=S in the range of 174.02–174.64 ppm. The 4-oxo-1,3-thiazolidine **16–20** in ^{1}H NMR spectra showed characteristic signal for CH_2 group at δ 4.10–4.34 ppm. Beside this, in ^{13}C NMR spectra for these derivatives a signal for C=O group was observed at δ 171.88–172.89 ppm. All other signals for aliphatic, aromatic, rings of 1,2,3,6-tetrahydrobenzoyl, 1,2,3,6-tetrahydrophthaloyl and bicyclo[2.2.2]-oct-7-ene were observed in usual regions.

The crystal structure determination of **17** was undertaken in order to confirm the synthesis pathway and its assumed molecular structure. The X-ray structure analysis revealed that compound **17** crystallizes in centrosymmetric space group $P\,\bar{1}$ with two independent molecules in the asymmetric part of the unit cell, denoted as *A* and *B* in Fig. 2, in the same arbitrarily chosen absolute configuration *S* at C8 atom and *R* at C9 atom.

The bond distances and angles in molecules *A* and *B* of **17** are in normal ranges [58] and are comparable to the corresponding values observed in closely related structures with thiazolidin-4-one system, *e.g.* 2-imino-3-(2-nitrophenyl)-1,3-thiazolidin-4-one [59], (*Z*)-3-phenyl-2-(propan-2-yl-idene-hydrazono)thiazolidin-4-one [60] and 2-methyl-3-(4-oxo-3-phenyl-thiazolidin-2-ylidenamino)-4-(3*H*)-chinazolinon [61]. The thiazolidine ring is planar to within 0.043(4) Å in *A* and 0.050(4) Å in *B*. The molecules *A* and *B* differ in conformation of the phenyl substituent in relation to the thiazolidine system, as shown in Fig. 3. This

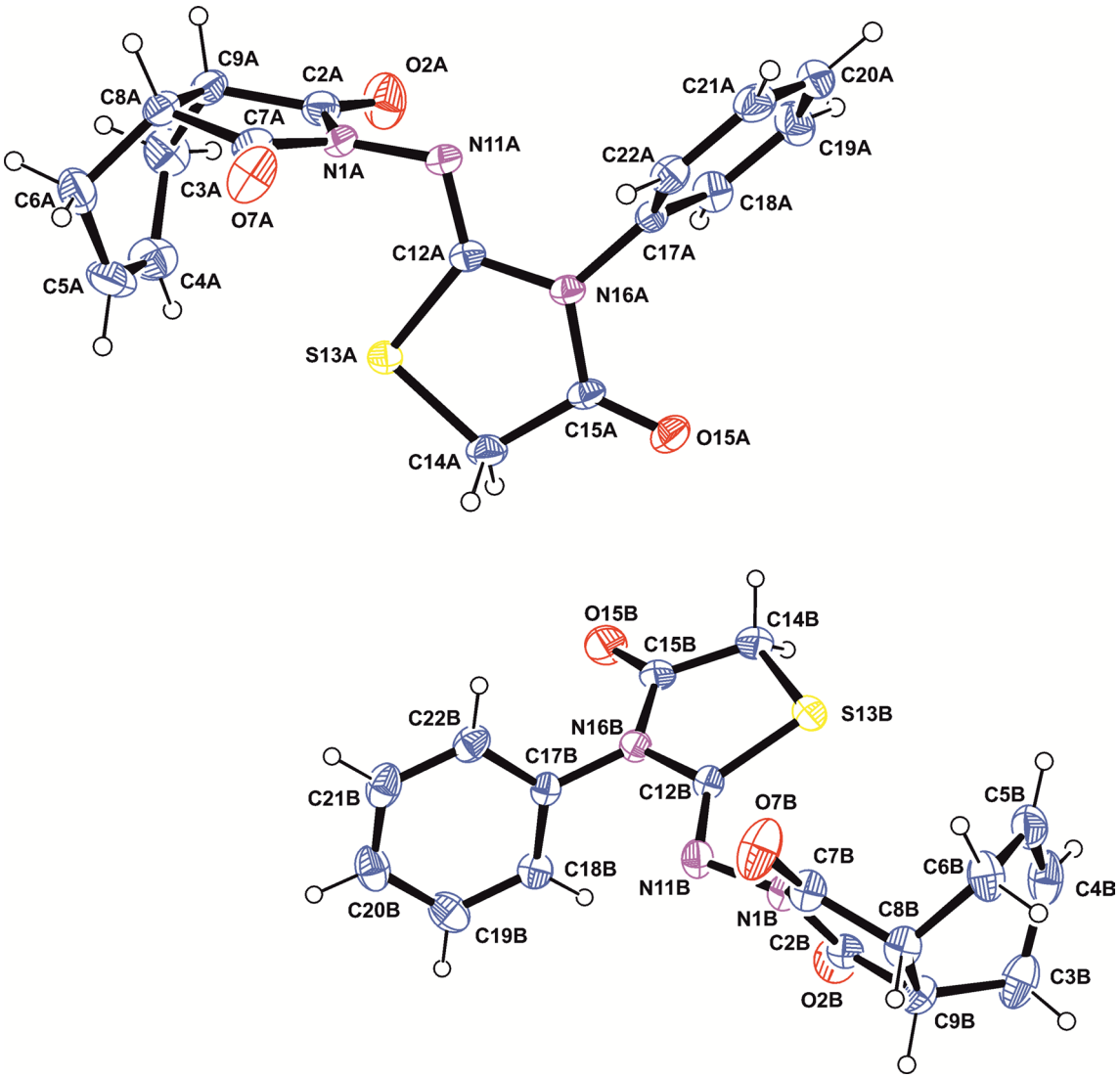


Fig. 2 ORTEP drawing of **17** at 50% probability

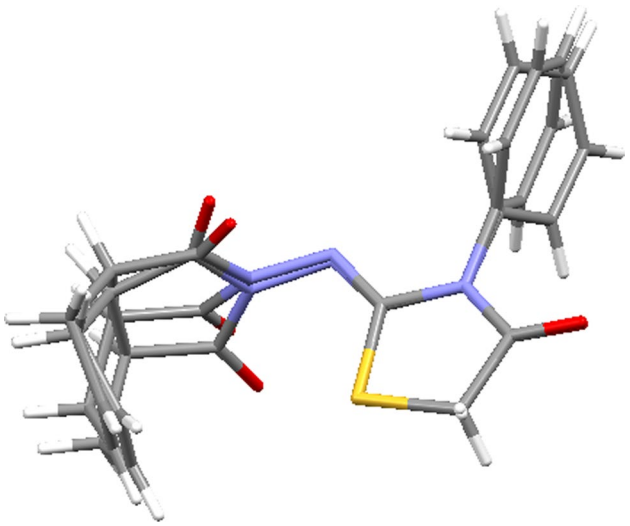


Fig. 3 Overlay of X-ray molecules *A* and *B* of compound **17** by least-squares fitting of the atoms of thiazolidine systems (RMS = 0.0324 Å)

conformation is described by the torsion angle C12-N16-C17-C18 of 96.3(4) and 77.6(5)° in *A* and *B*, respectively. The *cis-gauche* position of the 3a,4,7,7a-tetrahydroisoindole-1,3-dione system with respect to the thiazolidine ring is very similar in both molecules as shown by the torsion angles S13-C12-N11-N1 of 4.6(5)° in *A* and 5.7(5)° in *B* and C12-N11-N1-C2 of 99.4(4)° in *A* and 88.3(5)° in *B*. The 3a,4,7,7a-tetrahydroisoindole-1,3-dione fused system of **17** comprises a pyrolidine ring and partially saturated cyclohexene ring. The pyrolidine ring is planar to within 0.014(4) Å in *A* and 0.061(5) Å in *B*. The cyclohexene ring adopts the boat conformation in both molecules *A* and *B* with asymmetry parameters ΔC_S^{C3} of 2.3(5) and 10.4(4)° and $\Delta C_S^{C4,C5}$ of 2.7(6) and 10.1(5)° in *A* and *B*, respectively [62].

In the crystal structure of **17**, molecules form a three-dimensional network through the weak C–H…*X* (*X* = S, O) intermolecular hydrogen bonds (Table 1). The inversion-related molecules *A*(*x*, *y*, *z*) and *B*(1 − *x*, 1 − *y*, 1-*z*) and molecules *A*(*x*, *y*, *z*) and *B*(1 − *x*, 1 − *y*, 1 − *z*) form molecular trimers via C14A-H…S13B and C14B-H…O7B hydrogen bonds. These trimers are linked into molecular chains parallel to X crystallographic axis through C14A–H…O15B (− 1 + *x*, *y*, *z*) hydrogen bond.

Table 1 Hydrogen-bond geometry (Å, °)

D–H…A	D–H	H…A	D…A	D–H…A
C14A–H14A…S13B^{i}	0.97	2.73	3.636(5)	155
C14B–H14D…O7B^{i}	0.97	2.33	3.135(6)	140
C14A–H14B…O151B^{ii}	0.97	2.40	3.256(6)	147

(*i*) = 1 − *x*, 1 − *y*, 1 − *z*; (*ii*) = − 1 + *x*, *y*, *z*

Biological activity

The obtained compounds **1–20** have been studied as potential antiviral agents against *Herpes simplex* virus type l and coxsackievirus B3, and revealed a high efficacy as inhibitors of virus reproduction. Acyclovir was used as a reference compound for HSV-1 and ribavirin for CVB3. The influence of **1–20** on the Vero and FaDu cell cultures after incubation for 48–72 h is presented in Table 2.

The CC_{50} of tested substances **1–7** and **10** ranged from 27.02 to 509.57 µg/mL in Vero and 8.85–275.87 µg/mL in FaDu cell lines. The lowest cytotoxicity against Vero was observed for compounds **1**, **2**, **5**, **7** (> 500 µg/mL) and against FaDu for compound **7** (275.87 µg/mL). The highest cytotoxicity was noted for compound **4** in both Vero and FaDu cell lines (CC_{50} = 27.02 and 8.85 µg/mL, respectively). On the other hand, the most interesting compound was N^4-(2-methylphenyl)-N^1-(2-carboxy-1,2,3,6-tetrahydrobenzoyl)-thiosemicarbazide (**3**). This derivative displayed high toxicity against human epithelial cells FaDu in comparison with small toxicity against normal animal cell line (Vero).

Compounds from group of triazoles **(11–15)** demonstrated higher cytotoxicity against the Vero line (21.75–344.50 µg/mL) compared to the FaDu line (336.54–473.25 µg/mL). In group 4-oxo-1,3-thiazolidine **(16–20)**, the highest

Table 2 Cytotoxicity of compounds **1–20**

Compounds	Cell line/CC_{50} (µg/mL)[a]	
	Vero	FaDu
1	>500	153.51 ± 34.99
2	509.57 ± 24.21	262.37 ± 53.26
3	466.80 ± 29.79	10.37 ± 1.73
4	27.02 ± 4.18	8.85 ± 1.98
5	>500	240.60 ± 13.29
6	486.33 ± 23.67	270.35 ± 33.42
7	>500	275.87 ± 40.77
10	208.63 ± 50.82	99.58 ± 31.60
11	21.75 ± 6.86	473.25 ± 37.83
12	344.50 ± 20.51	336.54 ± 48.45
13	187.10 ± 9.33	396.74 ± 14.37
14	31.98 ± 1.03	341.06 ± 32.46
15	81.30 ± 3.12	461.00 ± 55.15
16	225.80 ± 32.27	330.15 ± 2.19
17	101.60 ± 32.53	274.20 ± 9.05
18	284.55 ± 51.41	500.00 ± 0.00
19	7.47 ± 0.67	23.98 ± 3.15
20	44.05 ± 12.03	38.43 ± 11.08

The CC_{50} is the cytotoxic concentration required to reduce the number of viable cells by 50%

[a]Mean ± SD values come from three independent experiments

cytotoxicity was found in compound **19**, both for Vero and FaDu lines, and CC_{50} were 7.47 and 23.98 µg/mL, respectively. Compound **18** was the least toxic compound in this group. CC_{50} was 284.55 µg/mL for Vero and 500 µg/mL for FaDu.

DMSO used as an eluent for the examined compounds in the tested concentration had no toxic effect on cell cultures. All compounds were evaluated for their cytotoxicity on cell line by a standard MTT assay.

Compounds were tested for in vitro antiviral activity using the cytopathic effect (CPE) inhibitory assay and by the virus titre reduction method. The results are presented in Table 3.

Noncytotoxic concentrations of 3.90, 15.62, 31.25, 62.50, 125 and 250 µg/mL were used for testing the antiviral activity of compounds.

The preliminary screening revealed the antiviral activity of compounds **3** and **4** from thiosemicarbazide group, both against HSV-1 and CVB3. Further determination of $TCID_{50}$ by the virus titre reduction method demonstrated that compound **3** in the concentration of 125 and 250 µg/mL influenced the HSV-1 replication by reducing the virus replication level by 3.45 log and 4.29 log, which resulted in reducing the titre by 40.5 and 50.04%, respectively. Compound **4** in all tested concentrations (15.62 and 31.25 µg/mL) demonstrated the antiviral activity, too. This derivative caused the decrease in the titre of HSV-1 by 3.03 log and 3.95 log, respectively (which corresponds to the following percentage levels of inhibition: 33.6 and 46.4%).

Compounds **3** and **4** only slightly reduced the CVB3 replication.

Other compounds did not demonstrate any significant antiviral activity. Thus, there is a need of further research to estimate their influence on different viruses. Prospectively, study of the mechanisms underlying their antiviral activity is necessary for the development of antiviral therapeutics to treat patients infected with HSV-1 and CVB3.

Table 3 Antiviral activity of the compounds against HSV-1 and CVB3

Compounds	Concentration (µg/mL)	HSV-1		CVB3	
		Antiviral activity	($TCID_{50}$/mL)[a]	Antiviral activity	($TCID_{50}$/mL)[a]
1	250	–	ND	–	ND
2	250	–	ND	–	ND
3	125	+	5.07 ± 0.41	+	9.24 ± 0.71
	250	+	4.23 ± 1.50	+	8.72 ± 0.88
4	15.62	+	5.49 ± 2.12	+	8.20 ± 1.41
	31.25	+	4.57 ± 0.76	+	8.50 ± 0.00
5	250	–	ND	–	ND
6	250	–	ND	–	ND
7	250	–	ND	–	ND
10	250	–	ND	–	ND
11	15.62	–	ND	–	ND
12	250	–	ND	–	ND
13	125	–	ND	–	ND
14	31.25	–	ND	–	ND
15	62.50	–	ND	–	ND
16	125	–	ND	–	ND
17	125	–	ND	–	ND
18	250	–	ND	–	ND
19	3.90	–	ND	–	ND
20	31.25	–	ND	–	ND
Acyclovir	250	+	0	ND	ND
Ribavirin	125	ND	ND	+	0
Virus control	0	+	8.52 ± 1.67	+	9.06 ± 1.69

Data represent the mean ± SD of at least 2–3 independent experiments

"–" negative antiviral activity in the screening assay

"+" positive antiviral activity in the screening assay

ND not determined

[a]The virus titres are shown in log. $TCID_{50}$—50% tissue culture infectious dose

Using methods outlined in the previous section, inhibition of growth by examined compounds: **2–7** and **14** for the reference strains of bacteria and fungi belonging to *Candida* spp. was shown. The results of our study presented in Table 4 indicated that compound **4** showed both the highest effect and the widest spectrum of activity against all Gram-positive bacteria, *Bordetella* spp. belonging to Gram-negative bacteria and fungi. This compound indicated especially very strong activity against *S. epidermidis* ATCC 12228 with MIC = 7.81 μg/mL and strong activity towards *S. aureus* ATCC 43300. The bioactivity against remaining reference staphylococci and micrococci was good (MIC = 31.25–62.5 μg/mL).

Moreover, substance **4** exhibited moderate or mild activity (MIC = 125–250 μg/mL) towards *Bacillus* spp. ATTC. *Bordetella bronchiseptica* ATTC 4617 from Gram-negative bacteria was also sensitive to **4** (MIC = 500 μg/mL and MBC > 1000 μg/mL). The activity towards all these bacteria was bacteriostatic, except micrococci (bactericidal effect with MBC/MIC = 2). Besides, this compounds exhibited fungicidal activity towards *Candida* spp. ATTC (MIC = 500 μg/mL and MFC = 500–1000 μg/mL, MFC/MIC = 1–2).

On the basis of minimal inhibitory concentration values, it was shown that other compound **3** had also wide spectrum of antimicrobial activity against Gram-positive bacteria and yeasts. The bactericidal effect of this substance was good against staphylococci, micrococci and *Bacillus subtilis* ATTC 6633 (MIC = 62.5–125 μg/mL and MBC = 62.5–250 μg/mL, MBC/MIC = 1–4) and moderate towards *Bacillus cereus* ATTC 10876 (MIC = 500 μg/mL and MBC = 1000 μg/mL, MBC/MIC = 2). Compound

Table 4 Activity data expressed as MIC (MBC/MFC) (μg/mL) against the reference strains of microorganisms

Species/compound	MIC (MBC/MFC) (μg/mL) of the tested compounds							CIP/FLU[a]
	2	3	4	5	6	7	14	
Gram-positive bacteria								
Staphylococcus aureus ATCC 25923	–	**62.5** (**125**)	**62.5** (> 1000)	–	–	–	–	0.488
Staphylococcus aureus ATCC 6538	–	**125** (**500**)	**31.25** (> 1000)	–	–	–	–	0.244
Staphylococcus aureus ATCC 43300	–	**62.5** (**125**)	**15.62** (> 1000)	1000 (> 1000)	–	–	–	0.244
Staphylococcus epidermidis ATCC 12228	–	**62.5** (**62.5**)	**7.81** (> 1000)	1000 (> 1000)	1000 (> 1000)	–	–	0.122
Micrococcus luteus ATCC 10240	1000 (> 1000)	**62.5** (**62.5**)	**31.25** (**62.5**)	1000 (> 1000)	1000 (> 1000)	100 (> 1000)	1000 (> 1000)	0.976
Bacillus subtilis ATCC 6633	1000 (> 1000)	**125** (250)	250 (> 1000)	1000 (> 1000)	1000 (> 1000)	–	–	0.030
Bacillus cereus ATCC 10876	–	500 (1000)	125 (> 1000)	–	–	–	–	0.061
Gram-negative bacteria								
Bordetella bronchiseptica ATCC 4617	–	–	500 (> 1000)	–	–	–	–	0.976
Fungi								
Candida albicans ATTC 10231	1000 (> 1000)	250 (500)	500 (1000)	–	500 (1000)	1000 (> 1000)	–	0.976[a]
Candida parapsilosis ATTC 22019	1000 (> 1000)	250 (1000)	500 (500)	–	1000 (1000)	–	–	1.953[a]

Bold values indicate compounds with significant activity

The standard chemotherapeutics agents: ciprofloxacin (CIT) or fluconazole (FLU[a]) used as positive control for bacteria and yeasts, respectively

No bioactivity – MIC > 1000 μg/mL; mild bioactivity – MIC = 501–1000 μg/mL; moderate bioactivity – MIC = 126–500 μg/mL; good bioactivity – MIC = 26–125 μg/mL; strong bioactivity – MIC = 10–25 μg/mL; very strong bioactivity – MIC < 10 μg/mL

3 exhibited also fungicidal activity towards *Candida* spp. ATTC (MIC = 250 μg/mL and MFC = 500–1000 μg/mL, MFC/MIC = 1–2) (Table 4).

The remaining synthesized compounds **2**, **5–7** and **14** had low activity on the single bacteria and fungi (MIC = 500–1000 μg/mL and MBC or MFC ≥ 1000 μg/mL) or had no inhibitory effect on the growth of microorganisms (Table 4).

In turn, compounds **1**, **8–13** and **15–20** indicated inactivity towards reference strains of bacteria and fungi.

Structure–activity relationship

It can be stated from the antiviral study that among obtained compounds, only two linear thiosemicarbazides **(3,4)** bearing 2-methylphenyl and 4-chlorophenyl groups attached to thiosemicarbazide at 4th position demonstrated the best activity against HSV-1 and CVB3. The antiviral activity in these derivatives is probably associated with the presence of a substituent attached to the phenyl ring at 2nd or 4th position, respectively, regardless of the character of substituent (electron donating or withdrawing). Other thiosemicarbazides with aliphatic or unsubstituted phenyl group were absolutely inactive. The dicarboximide and cyclic 1,2,4-triazole-5-thione and 4-oxo-1,3-thiazolidine had no any antiviral activity too. The cyclization reaction did not improve this activity. Similar results were obtained also for antimicrobial study. Compounds **3** and **4** presented the best antimicrobial activity too. According to this, it can be speculated that introduction of substituted phenyl group but only to thiosemicarbazide was the most beneficial in terms of both antiviral and antimicrobial activities.

Conclusions

In this paper, we synthesized and analysed four series of compounds: derivatives of thiosemicarbazide, dicarboximide, 1,2,4-triazole-5-thione and 4-oxo-1,3-thiazolidine. The reactions were carried out under moderately drastic conditions, and the solid products were isolated in medium and good yields. All obtained substances were examined for their antimicrobial and antiviral activity. The in vitro evaluation of cytotoxicity was also presented. According to our data, derivatives from thiosemicarbazide group presented the best biological activity. Among them, two compounds **3** and **4** showed a wide spectrum of antimicrobial activity against reference microorganisms and indicated both antibacterial and antifungal potential activities. The same derivatives **3** and **4** displayed the antiviral activity against HSV-1 and CVB3. The general observation was that compound **3**, N^4-(2-methylphenyl)-N^1-(2-carboxy-1,2,3,6-tetrahydrobenzoyl) thiosemicarbazide, is the most interesting due to its specific distinction between carcinoma and normal cells.

The obtained study can be applied in practice. On this basis, we are able to design novel biological active compounds which may be further modified and used as lead molecules for future investigation. Furthermore, this in turn will give hope for medicinal chemists as well as pharmacologists in the prediction of increased activity and allow to synthesize hitherto unknown substances exhibiting better antibacterial, antifungal and antiviral activities and then those reported in the above study. In this context, it is concluded that the research results make a great contribution to information on biological active compounds. In conclusion, these studies set the direction of further research.

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