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Synthesis and Antioxidant Activity of New Norcantharidin Analogs

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New norcantharidin analogs were designed and obtained as compounds with biological activity. As a starting material, *exo*-7-oxabicyclo[2.2.1]heptane-2,3-dicarboxylic acid anhydride was used. Three groups of compounds: dicarboximides, triazoles and thiazolidines were obtained in multistep reactions. The ^{1}H- and ^{13}C-NMR spectra were used to confirm the structures of all obtained products and they were in agreement with the proposed structure of substances. All derivatives were screened for their antioxidant activity. The most promising group was dicarboximides (**1**–**4**, **6**). Derivatives **2**–**4** displayed antioxidant activity with EC_{50} = 7.75–10.89 μg/ml, which may be comparable to strong antioxidant Trolox (EC_{50} = 6.13 μg/ml). Excellent activity with EC_{50} = 10.75 μg/ml also presented norcantharidin analog with 1,2,4-triazole system (**12**).

Keywords: norcantharidin analogs, heterocycles, cyclization reaction, antioxidant activity, structure–activity relationship, biological activity.

Introduction

Reactive oxygen species (ROS) are by-products of enzymatic reactions occurring in the organism. They are produced during endogenous processes such as cell respiration, phagocytosis, biosynthesis, catalysis and biotransformation. They can also be produced by exogenous processes (radiation, sunlight, heavy metals, bacteria, fungi, protozoa and viruses). Oxidative processes taking place under the influence of the ROS cause destruction of cells and tissue.[1] ROS are also very important factors in the aging processes, oxidative stress (OS) and in the pathogenesis of various diseases. Among these diseases are cancer, rheumatoid arthritis, various neurodegenerative and pulmonary diseases, atherosclerosis and DNA damage.[2,3] Probably, OS is the result of imbalance between production of free radicals and the speed of their neutralization in the body.[3,4] Antioxidants are natural or synthetic compounds, which play the shielding role against OS by reacting with free radicals, chelating catalytic metals and also by acting as oxygen scavengers.[5,6] Searching for the compounds, which better protect and prevent oxidative stress, is a very important issue in modern medicinal chemistry.

Norcantharidin (*exo*-7-oxabicyclo[2.2.1]heptane-2,3-dicarboxylic acid anhydride) is a terpenoid compound used as an anticancer drug in China. The first application of this compound was hepatoma, carcinomas of esophagus and gastric cardia.[7,8] In the recent years, special attention was paid to the mechanism of action of norcantharidin (NCTD), because it is complicated and still poorly elucidated. *Chang et al.* demonstrated that NCTD induced cytotoxic activity against HepG2 by apoptosis, which is mediated through reactive oxygen species (ROS) generation and mitochondrial pathway.[9] This anhydride also displayed proliferative activity against these cells.[10] Similar results were obtained for TSGH 8301 human urinary bladder carcinoma cells.[11] NCTD promoted ROS production and decreased levels of mitochondrial membrane potential. In the case of DU145 human prostate cancer cells, NCTD caused the reduction of expression of proliferating cell nuclear antigen, disor-

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der of mitochondrial membrane potential, deficit of ATP, induction of ROS and manganese superoxide dismutase, activation of adenosine 5′-monophosphate, which activated protein kinase and released cytochrome c. All of these factors led to death of DU145 cells by apoptosis.[12] Other mechanism of action was presented for NCTD by *Yeh et al.*[13] In this study, hepatocellular carcinoma (HCC) was used. NCTD inhibited expression of proteases MMP-9 and u-PA through the phosphorylation signaling pathway of ERK1/2 and NF- κB. Additionally, NCTD revealed a strong antimetastatic potential due to increased expression of degradation inhibitors ECM, which reduced the ability of cell mobility of Huh7.[13] NCTD is also active *in vitro* and *in vivo* against multiple myeloma through the NF-κB signal pathway.[14] Not only NCTD but also derivatives of NCTD presented anticancer activity. There are norcantharidin analogs with cyclic (lactone and imides ring) and with opening ring of the anhydride (amide, amide-acid and monoesterified analogs). All these derivatives have anticancer activity against liver carcinoma (HepG2 and Hep3B), colon cancer (HT29) and glioblastoma (SJ-G2).[15–17] Some of them are strong inhibitors of protein phosphatases PP1 and PP2.[18,19] Recently, *Peksel et al.* examined some derivatives of NCTD and bridged perhydroisoindole for their antioxidant and radical scavenging activities.[5] Most of the derivatives presented significant antioxidant and radical scavenging activities. On the basis of the above mentioned studies, we can state that in the future NCTD and derivatives of NCTD may be used in the prevention and treatment of cancer and this can be important in the prevention of secondary cancers.[9]

In the light of the above research and according with our sustained interest in the application of different dicarboxylic acid anhydride, especially NCTD in the synthesis of biologically active compounds, we designed and obtained a new class of norcantharidin analogs such as dicarboximides, triazoles and thiazolidines. Study of the antioxidant activities of NCTD and its derivatives is very important, so we examined prepared derivatives for these activity.

The newly obtained compounds can be applied in practice. The modification of the structure of the parent derivatives may provide a promising direction to obtain new biologically active norcantharidin analogs. These studies can also help medicinal chemists, biochemists and pharmacologists anticipate superior activity and synthesize unknown compounds with improved biological effect compared to the derivatives obtained in this work.

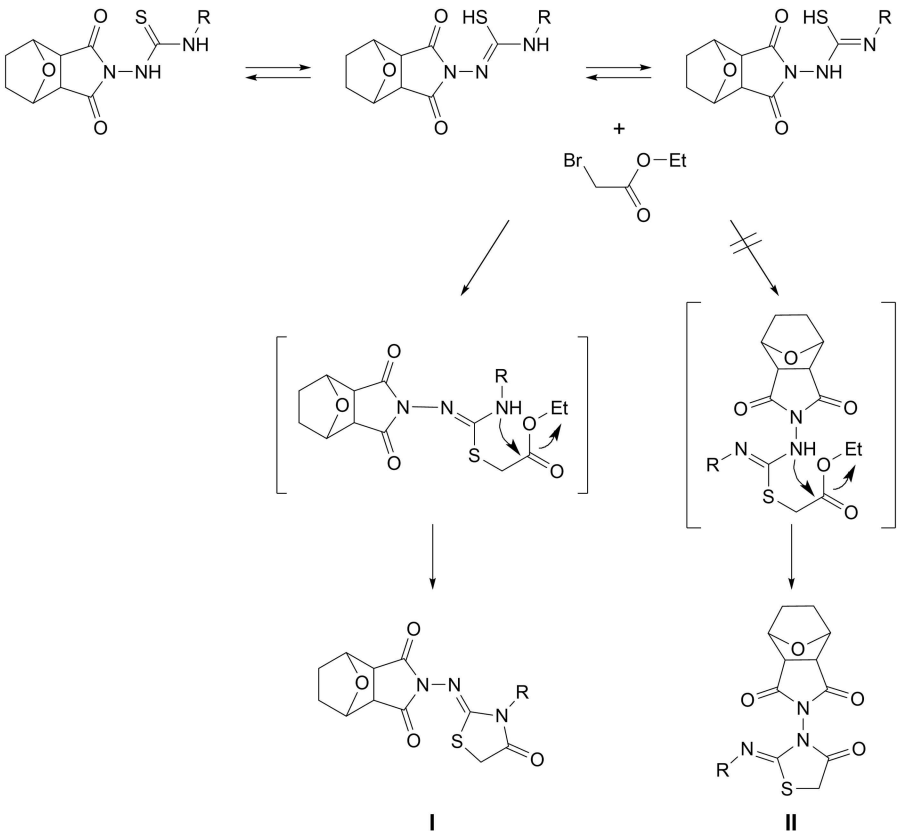
Results and Discussion

Chemistry

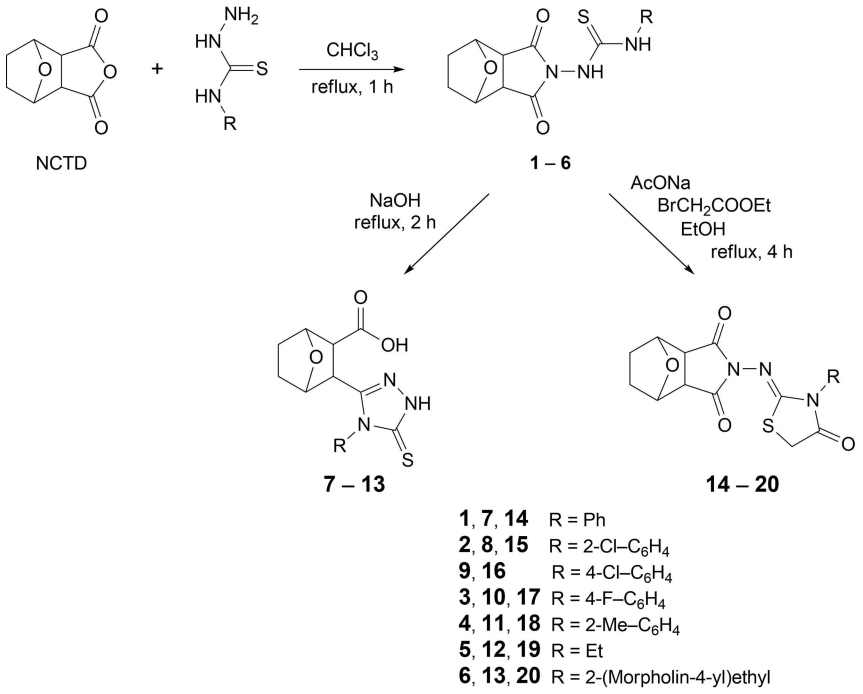
In the present work, *exo*-7-oxabicyclo[2.2.1]heptane-2,3-dicarboxylic acid anhydride (NCTD) was used as an initial material. This compound was heated with 4-substitued 3-thiosemicarbazide in dry chloroform and new *N*-(substituted-thioureido)aminobicyclo dicarboximides were synthesized. Intramolecular cyclization reaction of dicarboximide with sodium hydroxide led to formation of 3,4-disubstituted 1,2,4-triazoline-5-thione. In this reaction, the cyclic imide ring was opened[20] and then spontaneous cyclization of the linear chain of thiosemicarbazide took place, giving compounds with 1,2,4-triazole system.[15,21] Derivatives of 1,3-thiazolidine were prepared by Hantzsch reaction, in which the sulfur atom of thioureido group attacked the halogen atom of α-halocarbonyl compound and then elimination of hydrogen bromide and water led to the cyclic thiazole ring.[22] The thioureido group of dicarboximide, which has two nucleophilic centers, reacted with ethoxycarbonylmethyl group of ethyl bromoacetate, and then, intermediate for further cyclization was produced. The reaction proceeded in one step without isolation of intermediate.[23] Two different structural isomers **I** and **II** can be obtained in this synthesis (*Scheme 1*). Based on the previous article, it can be speculated that only one structural isomer **I** was obtained.[24] In conclusion, the structures of the products **14–20** were determined based on previous discussion of the structures of similar compounds[24] and results are consistent with the description in literature.[23,25–27] The synthetic pathway is presented in *Scheme 2*.

Unfortunately, in the case of the reaction of 4-(4-chlorophenyl)-3-thiosemicarbazide with NCTD, pure dicarboximide was not isolated, but further cyclization of this derivative led to 1,2,4-triazole (**9**) and 1,3-thiazolidine (**16**) with good purity. 1-(1,3-Dioxooctahydro-2*H*-4,7-epoxyisoindol-2-yl)-3-phenylthiourea (**1**) was previously obtained by other authors.[28] The spectral characterization of compound **1**, which we have synthesized, is consistent with the published data.

The ^{1}H- and ^{13}C-NMR spectra were used to confirm the structures of all the obtained products and they were in agreement with the proposed structure of substances **1–20**. The ^{1}H-NMR spectra of compounds **1–6** presented two singlets for two NH groups between 8.13–9.91 ppm and 9.80–10.37 ppm. In the ^{13}C-NMR spectra of these compounds (**1–6**), typical signals corresponding to cyclic carboximides C=O



Scheme 1. Probable mechanism leading to the formation of 1,3-thiazolidine.



Scheme 2. Synthesis of compounds **1**–**20**.

groups and C=S group of linear chain were observed. They resonated between 174.61–175.20 ppm and 180.98–181.78 ppm, respectively. The 1,2,4-triazole-5-thiones (**7**–**12**) in the ^{1}H-NMR spectra presented characteristic singlet signal for the NH group at 11.97–12.35 ppm. In the ^{13}C-NMR spectra for these derivatives, the signal corresponding to the C=S group appeared at 171.71–171.96 ppm. In the ^{1}H-NMR spectra for 1,3-thiazolidine (**14**–**20**), characteristic signals due to the CH_2 protons appeared as singlet at 4.14–4.36 ppm, whereas ^{13}C-NMR presented signals due to the C=O group of thiazolidine ring at 171.29–172.40 ppm.

Antiradical Activity

All the synthesized compounds were primary screened for their antioxidant activity using DPPH-TLC assay. The most active derivatives **1**–**10**, **12**, **14**–**18** and **20** were selected for antiradical activity analysis using an improved $ABTS^{+\bullet}$ decolorization assay. Among all examined substances, the most promising group were dicarboximides (**1**–**4**, **6**), whereas compounds with 1,3-thiazolidine moiety (**14**–**18**, **20**) were inactive. Excellent antioxidant activity was displayed by dicarboximides **2**–**4** with EC_{50} = 7.75–10.89 μg/ml, which may be comparable to strong antioxidant Trolox (EC_{50} = 6.13 μg/ml). The dicarboximide derivatives **1** and **6** possess quite good activity with EC_{50} = 24.83–25.35 μg/ml. Only the dicarboximide **5** bearing ethyl substituent was inactive. Interestingly, derivative **12** with ethyl substituent at 4-position of triazole ring, which was obtained from the inactive compound **5**, also presented excellent activity with EC_{50} = 10.75 μg/ml. The other 1,2,4-triazoles **7**–**10** indicated good antioxidant activity (EC_{50} = 15.52–25.35 μg/ml). With regards to chelating power (CHEL), the highest activity showed compound **6**, but only 36% inhibition was determined. The remaining compounds were inactive. The antiradical activity of synthesized compounds (**1**–**20**) is presented in *Table 1*.

Some derivatives of NCTD and bridged perhydroisoindole were also evaluated for their antioxidant and radical scavenging activities by *Peksel et al.*[5] Examined compounds presented lower radical scavenging activities (IC_{50} = 25.6–130.6 μg/ml) when compared to commercial antioxidants, for example Trolox (IC_{50} = 4.5 μg/ml).

Table 1. Antiradical activity of synthesized compounds **1**–**20**.

Compound	Antioxidant activity		
	$DPPH^{\bullet}$ EC_{50} (range from 0 to 5) 0 – inactive, 5 – very active	$ABTS^{+\bullet}$ TEAC [mM Trolox g^{-1} compound][a]	EC_{50} [μg mL^{-1}][a]
1	5	0.989 ± 0.025	24.83 ± 0.62
2	4	2.750 ± 0.033	8.93 ± 0.11
3	5	2.256 ± 0.095	10.89 ± 0.46
4	4	3.173 ± 0.140	7.75 ± 0.35
5	2	0.163 ± 0.009	150.49 ± 7.81
6	3	0.969 ± 0.029	25.35 ± 0.77
7	4	1.303 ± 0.049	18.86 ± 0.71
8	2	1.444 ± 0.046	17.02 ± 0.53
9	4	1.582 ± 0.017	15.52 ± 0.17
10	3	1.665 ± 0.036	14.75 ± 0.32
11	nd	nd	nd
12	3	2.284 ± 0.056	10.75 ± 0.27
13	nd		
14	0/1	–	–
15	0/1	–	-
16	0/1	–	–
17	0/1	–	–
18	0/1	–	–
19	nd	nd	nd
20	0/1	–	–

[a] Values are expressed as means of three replicates ± SD. '–', no activity; 'nd', not determined; EC_{50} for Trolox = 6.13 ± 0.28 μg mL^{-1}.

Structure-Activity Relationship

From the antiradical activity analysis, it can be stated that the initial compound, *exo*-7-oxabicyclo[2.2.1]-heptane-2,3-dicarboxylic acid anhydride (NCTD), had no antioxidant activity, whereas the dicarboximide derivative obtained from NCTD and various 4-substituted 3-thiosemicarbazides indicated good to excellent activity. Among them, the most active were compounds **2**–**4** with aryl substituent (2-methylphenyl, 2-chlorophenyl and 4-fluorophenyl) attached to nitrogen of thioureido moiety. Compound **1** with unsubstituted phenyl group attached to nitrogen of thioureido moiety displayed only good activity. In the case of dicarboximide derivatives with aromatic substituents, antioxidant activity can be connected with the presence of substituents attached to phenyl moiety at 2- or 4-position. It can be stated that the character of substituent (electron donating or withdrawing) does not affect activity. Dicarboximide derivatives with aliphatic substituents: ethyl (in **5**) or morpholinylethyl (in **6**) at nitrogen of thioureido

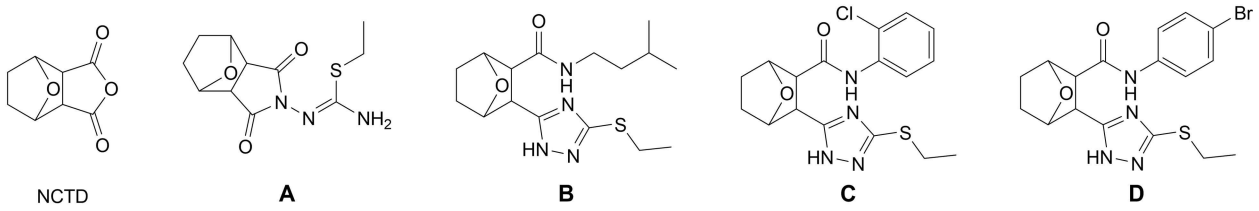


Figure 1. Structure of compounds additionally examined for antiradical activity.

moiety had no activity or presented good activity, respectively. Closer inspection of these compounds revealed that better activity showed derivative **6** with a bulky aliphatic substituent. Interestingly, when corresponding inactive dicarboximide bearing ethyl substituent was cyclized to 3,4-disubstituted 1,2,4-triazoline-5-thione, the obtained compound **12** revealed excellent antiradical activity. In this case, the cyclization reaction improved activity. Derivative **7** with unsubstituted phenyl at 4-position of 1,2,4-triazoline-5-thione system in comparison to dicarboximide **1** with the same substituent showed improved activity, too. Three other 1,2,4-triazoline-5-thione derivatives **8**–**10** did not improve their activity in comparison to counterpart dicarboximides **3**–**4**.

For a more accurate SAR analysis, some compounds with a similar structure, which were obtained previously as anticancer agents, were also tested.[29] Structures of these derivatives is presented in *Figure 1*. Among them, there were NCTD, ethyl *N*-(1,3-dioxooctahydro-2*H*-4,7-epoxyisoindol-2-yl)carbamimidothioate (**A**) and *N*-substituted amides of 3-[3-(ethylsulfanyl)-1*H*-1,2,4-triazol-5-yl]-7-oxabicyclo[2.2.1]heptane-2-carboxylic acid (**B**–**D**).

In the case of 1,2,4-triazoles (**B**–**D**) with the amide and ethylsulfanyl group from *Figure 1*, no antiradical activity was observed, whereas 1,2,4-triazoline-5-thione **7**–**10** and **12** with carboxylic and thione group showed good to excellent activity. This fact suggested that the carboxylic group and thione form of triazole were beneficial in terms of bioactivity. In general, the introduction of unsubstituted phenyl, methylphenyl, chlorophenyl or fluorophenyl substituent to dicarboximide and 1,2,4-triazoline-5-thione is also beneficial in terms of antiradical activity. Unfortunately, in the case of 1,3-thiazolidine derivatives (**14**–**18**, **20**), probably, the presence of thiazolidine moiety (five-membered heterocyclic ring with two heteroatoms of sulfur and nitrogen) can be connected with lack of antiradical activity irrespectively of substituent. All these results provide useful information about structure–activity relationships.

Conclusions

In this work, 20 analogs of norcantharidin were obtained. Among them, there were dicarboximides (**1**–**6**), triazoles (**7**–**13**) and thiazolidines (**14**–**20**). All the synthesized compounds were evaluated for their antioxidant activity. Generally, new triazoles (**7**–**13**) improved their antiradical activity compared to the derivatives obtained previously.[5] The introduction of the carboxylic group into the triazole ring increased the activity. The most promising group were dicarboximides, especially compounds **2**–**4** with EC_{50} = 7.75–10.89 μg/ml. Similar activity with EC_{50} = 10.75 μg/ml displayed 3-(4-ethyl-4,5-dihydro-5-thioxo-1*H*-1,2,4-triazol-3-yl)-7-oxabicyclo[2.2.1]heptane-2-carboxylic acid (**12**). The antioxidant activity of these compounds was comparable to commonly used antioxidant Trolox. Derivatives with the best antiradical activity may be a promising lead for future investigation. Additionally, the most active compounds, dicarboximides (**2**–**4**) and triazole **12**, may be promising candidates for therapeutic applications.

Experimental Section

Chemistry

All the used chemicals were purchased from Sigma-Aldrich (Munich, Germany) or Merck Co. (Darmstadt, Germany) and applied without further purification. Melting points were determined in a Fischer-Johns block and presented without any corrections. ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker Avance 300 MHz spectrometer in (D_6)DMSO; δ in ppm relative to Me_4Si as internal standard, *J* in Hz. MS spectra were recorded on Bruker microTOF-Q II and processed using Compass Data Analysis software; in *m/z*. Elemental analysis was made on a PerkinElmer 2400 CHN analyzer. All results were in good agreement with calculated values. The error range of ±0.4% was for each element analyzed.

General Method for the Synthesis of N-*(Substituted-thioureido)aminobicyclo Dicarboximide* (**1**–**6**). 10 mmol of *exo*-7-oxabicyclo[2.2.1]heptane-2,3-dicarboxylic acid 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 or ethanol-water.

1-(1,3-Dioxooctahydro-2*H*-4,7-epoxyisoindol-2-yl)-3-phenylthiourea (**1**). Yield: 80%. M.p. 190–192 °C. ^{1}H-NMR (300 MHz, (D$_6$)DMSO): 1.66–1.73 (*m*, 2CH$_2$); 3.17 (*s*, 2CH); 4.75 (*s*, CH–O–CH); 7.17–7.36 (*m*, Ph); 9.91 (br. *s*, NH); 10.22 (*s*, NH). ^{13}C-NMR (75 MHz, (D$_6$)DMSO): 28.52 (2CH$_2$); 48.68 (2CH); 78.93 (CH–O–CH); 125.90 (2 *o*-PhCH); 126.13 (*p*-PhCH); 128.91 (2 *m*-PhCH); 138.99 (*i*-PhC); 174.74 (2C=O); 181.21 (C=S). HR-MS: 318.0597 ([*M* + H]$^+$, $C_{15}H_{15}N_3O_3S^+$; calc. 317.3629).

1-(2-Chlorophenyl)-3-(1,3-dioxooctahydro-2*H*-4,7-epoxyisoindol-2-yl)thiourea (**2**). Yield: 86%. M.p. 186–188 °C. ^{1}H-NMR (300 MHz, (D$_6$)DMSO): 1.70 (*s*, 2CH$_2$); 3.15 (*s*, 2CH); 4.75 (*s*, CH–O–CH); 7.18–7.64 (*m*, Ph); 9.83 (br. *s*, NH); 10.37 (*s*, NH). ^{13}C-NMR (75 MHz, (D$_6$)DMSO): 28.52 (2CH$_2$); 48.90 (2CH); 78.97 (CH–O–CH); 127.87 (*o*-PhCH); 129.20 (*p*-PhCH); 130.00 (*m*-PhCH); 131.85 (*m*-PhCH); 132.22 (*o*-Ph*C*–Cl); 136.39 (*i*-PhC); 174.61 (2C=O); 181.78 (C=S). HR-MS: 352.0218 ([*M* + H]$^+$, $C_{15}H_{14}ClN_3O_3S^+$; calc. 351.8080).

1-(1,3-Dioxooctahydro-2*H*-4,7-epoxyisoindol-2-yl)-3-(4-fluorophenyl)thiourea (**3**). Yield: 95%. M.p. 174–176 °C. ^{1}H-NMR (300 MHz, (D$_6$)DMSO): 1.53–1.83 (*m*, 2CH$_2$); 3.17 (*s*, 2CH); 4.79 (*s*, CH–O–CH); 7.03–7.68 (*m*, Ph); 9.90 (br. *s*, NH); 10.27 (*s*, NH). ^{13}C-NMR (75 MHz, (D$_6$)DMSO): 28.52 (2CH$_2$); 48.71 (2CH); 78.95 (CH–O–CH); 115.49 (2 *m*-PhCH); 126.74 (*o*-PhCH); 129.07 (*o*-PhCH); 135.26 (*i*-PhC); 158.68 (*p*-Ph*C*–F); 161.89 (*p*-Ph*C*–F); 174.75 (2C=O); 181.54 (C=S). HR-MS: 336.0531 ([*M* + H]$^+$, $C_{15}H_{14}FN_3O_3S^+$; calc. 335.3534).

1-(1,3-Dioxooctahydro-2*H*-4,7-epoxyisoindol-2-yl)-3-(2-methylphenyl)thiourea (**4**). Yield: 83%. M.p. 200–202 °C. ^{1}H-NMR (300 MHz, (D$_6$)DMSO): 1.64–1.76 (*m*, 2CH$_2$); 2.13 (br. *s*, Me), 3.15 (*s*, 2CH); 4.75 (*s*, CH–O–CH); 7.06–7.28 (*m*, Ph); 9.68 (br. *s*, NH); 10.21 (*s*, NH). ^{13}C-NMR (75 MHz, (D$_6$)DMSO): 17.87 (Me); 28.53 (2CH$_2$); 48.89 (2CH); 78.93 (CH–O–CH); 126.64 (*o*-PhCH); 127.73 (*p*-PhCH); 129.29 (*m*-PhCH); 130.79 (*m*-PhCH); 136.30 (*o*-Ph*C*–Me); 137.51 (*i*-PhC); 174.91 (2C=O); 181.15 (C=S). HR-MS: 332.0755 ([*M* + H]$^+$, $C_{16}H_{17}N_3O_3S^+$; calc. 331.3895).

1-(1,3-Dioxooctahydro-2*H*-4,7-epoxyisoindol-2-yl)-3-ethylthiourea (**5**). Yield: 93%. M.p. 170–172 °C. ^{1}H-NMR (300 MHz, (D$_6$)DMSO): 1.03 (*t*, *J* = 6.9, Me); 1.65–1.72 (*m*, 2CH$_2$); 3.11 (*s*, 2CH); 3.42 (*q*, *J* = 6.9, *CH*$_2$Me); 4.71 (*s*, CH–O–CH); 8.22 (br. *s*, NH); 9.80 (*s*, NH). ^{13}C-NMR (75 MHz, (D$_6$)DMSO): 14.27 (Me); 28.40 (2CH$_2$); 38.20 (*CH*$_2$Me); 48.51 (2CH); 79.08 (CH–O–CH); 175.20 (2C=O); 180.98 (C=S). HR-MS: 270.0691 ([*M* + H]$^+$, $C_{11}H_{15}N_3O_3S^+$; calc. 269.3201).

1-(1,3-Dioxooctahydro-2*H*-4,7-epoxyisoindol-2-yl)-3-[2-(morpholin-4-yl)ethyl]thiourea (**6**). Yield: 87%. M.p. 180–182 °C. ^{1}H-NMR (300 MHz, (D$_6$)DMSO): 1.59 (*s*, 2CH$_2$); 2.38 (*s*, CH$_2$–N–CH$_2$ + CH$_2$); 3.12 (*s*, 2CH); 3.58 (*s*, CH$_2$–O–CH$_2$ + CH$_2$); 4.72 (*s*, CH–O–CH); 8.13 (br. *s*, NH); 9.92 (*s*, NH). ^{13}C-NMR (75 MHz, (D$_6$)DMSO): 28.48 (2CH$_2$); 41.78 (CH$_2$); 48.57 (2CH); 53.77 (CH$_2$–N–CH$_2$); 56.86 (CH$_2$); 66.60 (CH$_2$–O–CH$_2$); 78.98 (CH–O–CH); 174.84 (2C=O); 181.50 (C=S). HR-MS: 353.0206 ([*M* + H]$^+$, $C_{15}H_{22}N_4O_4S^+$; calc. 354.4246).

General Method for the Synthesis of 3,4-Disubstituted 1,2,4-Triazoline-5-thione (**7**–**13**). A solution of *N*-(substituted-thioureido)aminobicyclo dicarboximide (2 mmol) in 2% sodium hydroxide (5 ml) was refluxed for 2 h. After cooling, the mixture was filtered off and the isolated solution was acidified with 3 M HCl. The precipitate was filtered off and crystallized from ethanol.

3-(4,5-Dihydro-4-phenyl-5-thioxo-1*H*-1,2,4-triazol-3-yl)-7-oxabicyclo[2.2.1]heptane-2-carboxylic Acid (**7**). Yield: 70%. M.p. 260–262 °C. ^{1}H-NMR (300 MHz, (D$_6$)DMSO): 1.37–1.56 (*m*, 2CH$_2$); 3.41–3.45 (*m*, 2CH); 4.77 (*s*, CH–O–CH); 7.39–7.60 (*m*, Ph); 12.29 (br. *s*, NH); 13.63 (*s*, COOH). ^{13}C-NMR (75 MHz, (D$_6$)DMSO): 28.21 (CH$_2$); 29.63 (CH$_2$); 43.50 (*CH*-triazole); 53.87 (*CH*–COOH); 78.02 (CH–O–CH); 79.72 (CH–O–CH); 129.14 (2 *o*-PhCH); 129.77 (2 *m*-PhCH + *p*-PhCH); 134.27 (*i*-PhC); 152.78 (C$_{triazole}$); 167.31 (COOH); 171.77 (C=S). HR-MS: 318.0613 ([*M* + H]$^+$, $C_{15}H_{15}N_3O_3S^+$; calc. 317.3629).

3-[4-(2-Chlorophenyl)-4,5-dihydro-5-thioxo-1*H*-1,2,4-triazol-3-yl]-7-oxabicyclo[2.2.1]heptane-2-carboxylic Acid (**8**). Yield: 65%. M.p. 260–262 °C. ^{1}H-NMR (300 MHz, (D$_6$)DMSO): 1.29–1.66 (*m*, 2CH$_2$); 3.45–3.47 (*m*, 2CH); 4.80 (*s*, CH–O–CH); 7.29–7.86 (*m*, Ph); 12.30 (br. *s*, NH); 13.76 (*s*, COOH). ^{13}C-NMR (75 MHz, (D$_6$)DMSO): 28.16 (CH$_2$); 29.59 (CH$_2$); 43.53 (*CH*-triazole); 54.12 (*CH*–COOH); 77.70 (CH–O–CH); 79.88 (CH–O–CH); 128.92 (*o*-PhCH); 130.97 (*p*-PhCH); 131.73

(*o*-PhC–Cl); 132.09 (*m*-PhCH); 132.28 (*m*-PhCH); 132.81 (*i*-PhC); 152.82 ($C_{triazole}$); 167.32 (COOH); 171.71 (C=S). HR-MS: 352.0228 ($[M+H]^+$, $C_{15}H_{14}ClN_3O_3S^+$; calc. 351.8080).

3-[4-(4-Chlorophenyl)-4,5-dihydro-5-thioxo-1*H*-1,2,4-triazol-3-yl]-7-oxabicyclo[2.2.1]heptane-2-carboxylic Acid (**9**). Yield: 62%. M.p. 160–162 °C. ^{1}H-NMR (300 MHz, (D_6)DMSO): 1.30–1.67 (*m*, $2CH_2$); 2.77–3.12 (*m*, 2CH); 4.78 (*s*, CH–O–CH); 7.44–7.65 (*m*, Ph); 12.35 (br. *s*, NH); 13.68 (*s*, COOH). ^{13}C-NMR (75 MHz, (D_6)DMSO): 28.14 (CH_2); 29.62 (CH_2); 43.42 (*CH*-triazole); 53.96 (*CH*–COOH); 77.67 (CH–O–CH); 80.37 (CH–O–CH); 129.88 (2 *o*-PhCH); 131.10 (2 *m*-PhCH); 133.16 (*p*-PhC–Cl); 134.48 (*i*-PhC); 152.83 ($C_{triazole}$); 167.28 (COOH); 171.86 (C=S). HR-MS: 352.0228 ($[M+H]^+$, $C_{15}H_{14}ClN_3O_3S^+$; calc. 351.8080).

3-[4-(4-Fluorophenyl)-4,5-dihydro-5-thioxo-1*H*-1,2,4-triazol-3-yl]-7-oxabicyclo[2.2.1]heptane-2-carboxylic Acid (**10**). Yield: 72%. M.p. 245–247 °C. ^{1}H-NMR (300 MHz, (D_6)DMSO): 1.33–1.66 (*m*, $2CH_2$); 2.80–3.06 (*m*, 2CH); 4.78 (*s*, CH–O–CH); 7.32–7.58 (*m*, Ph); 12.25 (br. *s*, NH); 13.66 (*s*, COOH). ^{13}C-NMR (75 MHz, (D_6)DMSO): 28.16 (CH_2); 29.62 (CH_2); 43.43 (*CH*-triazole); 53.90 (*CH*–COOH); 78.05 (CH–O–CH); 79.72 (CH–O–CH); 116.58 (2*m*-PhCH); 116.89 (2 *m*-PhCH); 130.50 (*i*-PhC); 131.44 (*o*-PhCH); 131.57 (*o*-PhCH); 152.94 ($C_{triazole}$); 160,97 (*p*-PhC–F); 164,41 (*p*-PhC–F); 167.40 (COOH); 171.86 (C=S). HR-MS: 336.0524 ($[M+H]^+$, $C_{15}H_{14}FN_3O_3S^+$; calc. 335.3534).

3-[4,5-Dihydro-4-(2-methylphenyl)-5-thioxo-1*H*-1,2,4-triazol-3-yl]-7-oxabicyclo[2.2.1]heptane-2-carboxylic Acid (**11**). Yield: 68%. M.p. 168–170 °C. ^{1}H-NMR (300 MHz, (D_6)DMSO): 1.31–1.69 (*m*, $2CH_2$); 2.12 (*s*, Me); 3.40–3.47 (*m*, 2CH); 4.73 (*s*, CH–O–CH); 7.18–7.49 (*m*, Ph); 12.31 (br. *s*, NH); 13.69 (*s*, COOH). ^{13}C-NMR (75 MHz, (D_6)DMSO): 17.82 (Me); 28.09 (CH_2); 29.66 (CH_2); 43.41 (*CH*-triazole); 54.29 (*CH*–COOH); 77.66 (CH–O–CH); 79.84 (CH–O–CH); 127.65 (*o*-PhCH); 129.38 (*p*-PhCH); 130.44 (*m*-PhCH); 131.54 (*m*-PhCH); 133.12 (*o*-PhC–Me); 136.60 (*i*-PhC); 152.83 ($C_{triazole}$); 166.71 (COOH); 171.73 (C=S). HR-MS: 332.0791 ($[M+H]^+$, $C_{16}H_{17}N_3O_3S^+$; calc. 331.3895).

3-(4-Ethyl-4,5-dihydro-5-thioxo-1*H*-1,2,4-triazol-3-yl)-7-oxabicyclo[2.2.1]heptane-2-carboxylic Acid (**12**). Yield: 60%. M.p. 260–262 °C. ^{1}H-NMR (300 MHz, (D_6)DMSO): 1.21 (*t*, $J=6.0$, Me); 1.50–1.84 (*m*, $2CH_2$); 3.29–3.50 (*m*, 2CH); 3.88 (*q*, $J=6.0$, CH_2Me); 4.80 (*s*, CH–O–CH); 12.35 (br. *s*, NH); 13.39 (*s*, COOH). ^{13}C-NMR (75 MHz, (D_6)DMSO): 13.90 (Me); 28.34 (CH_2); 29.65 (CH_2); 38.51 (CH_2); 43.15 (*CH*-triazole); 53.89 (*CH*–COOH); 78.00 (CH–O–CH); 79.52 (CH–O–CH); 152.44 ($C_{triazole}$); 165.74 (COOH); 171.81 (C=S). HR-MS: 270.0658 ($[M+H]^+$, $C_{11}H_{15}N_3O_3S^+$; calc. 269.3201).

3-[4,5-Dihydro-4-[2-(4-morpholinyl)ethyl]-5-thioxo-1*H*-1,2,4-triazol-3-yl]-7-oxabicyclo[2.2.1]-heptane-2-carboxylic Acid (**13**). Yield: 66%. M.p. 256–258 °C. ^{1}H-NMR (300 MHz, (D_6)DMSO): 1.53 (*s*, $2CH_2$); 2.95–3.35 (*m*, CH_2); 3.36–3.52 (*m*, CH_2–N–CH_2); 3.68–3.71 (*m*, CH_2); 3.77–4.17 (*m*, CH_2–O–CH_2); 4.39–4.57 (*m*, 2CH); 4.90 (*s*, CH–O–CH); 11.97 (br. *s*, NH); 13.58 (*s*, COOH). ^{13}C-NMR (75 MHz, (D_6)DMSO): 28.51 (CH_2); 29.30 (CH_2); 38.02 (CH_2); 43.13 (*CH*-triazole); 50.68 (CH_2); 52.52 (CH_2–N–CH_2); 54.27 (*CH*–COOH); 63.74 (CH_2–O–CH_2); 78.05 (CH–O–CH); 79.55 (CH–O–CH); 153.07 ($C_{triazole}$); 165.05 (COOH); 171.96 (C=S). HR-MS: 355.1156 ($[M+H]^+$, $C_{15}H_{22}N_4O_4S^+$; calc. 354.4246).

General Method for the Synthesis of N-*[(4-Oxo-3-substituted-1,3-thiazolidin-2-yl)imino]-7-oxabicyclo-[2.2.1]heptane Dicarboximide* (**14**–**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 were added and the mixture was refluxed for 4 h. After cooling, the solution was allowed to stand overnight. The precipitate was filtered off and crystallized from ethanol.

2-[(4-Oxo-3-phenyl-1,3-thiazolidin-2-ylidene)-amino]hexahydro-1*H*-4,7-epoxyisoindole-1,3(2*H*)-dione (**14**). Yield: 86%. M.p. 282–284 °C. ^{1}H-NMR (300 MHz, (D_6)DMSO): 1.71 (*s*, $2CH_2$); 3.18 (*s*, 2CH); 4.33 (*s*, CH_2); 4.76 (*s*, CH–O–CH); 7.30–7.75 (*m*, Ph). ^{13}C-NMR (75 MHz, (D_6)DMSO): 28.43 ($2CH_2$); 33.61 ($CH_{2thiazol}$); 48.79 (2CH); 78.81 (CH–O–CH); 128.47 (2 *o*-PhCH); 129.38 (*p*-PhCH); 129.62 (2 *m*-PhCH); 134.70 (*i*-PhC); 170.81 ($C_{thiazol}$); 171.98 (C=O); 172.73 (2C=O). HR-MS: 358.0558 ($[M+H]^+$, $C_{17}H_{15}N_3O_4S^+$; calc. 357.3837).

2-{[3-(2-Chlorophenyl)-4-oxo-1,3-thiazolidin-2-ylidene]amino}hexahydro-1*H*-4,7-epoxyisoindole-1,3(2*H*)-dione (**15**). Yield: 62%. M.p. 238–240 °C. ^{1}H-NMR (300 MHz, (D_6)DMSO): 1.65 (*s*, $2CH_2$); 3.09 (*s*, 2CH); 4.36 (*s*, CH_2); 4.69 (*s*, CH–O–CH); 7.47–7.75 (*m*, Ph). ^{13}C-NMR (75 MHz, (D_6)DMSO): 28.43 ($2CH_2$); 33.59 ($CH_{2thiazol}$); 48.79 (2CH); 78.82 (CH–O–CH); 128.85 (*o*-PhCH); 130.52 (*p*-PhCH); 131.34 (*m*-PhCH); 131.86 (*m*-PhCH); 132.13 (*o*-PhC–Cl); 132.32 (*i*-PhC); 169.18

($C_{thiazol}$); 171.29 (C=O); 172.45 (2C=O). HR-MS: 392.0139 ($[M+H]^+$, $C_{17}H_{14}ClN_3O_4S^+$; calc. 391.8288).

2-{[3-(4-Chlorophenyl)-4-oxo-1,3-thiazolidin-2-ylidene]amino}hexahydro-1*H*-4,7-epoxyisoindole-1,3(2*H*)-dione (**16**). Yield: 65%. M.p. 278–280 °C. ^{1}H-NMR (300 MHz, (D_6)DMSO): 1.62 (*s*, $2CH_2$); 3.12 (*s*, 2CH); 4.22 (*s*, CH_2); 4.70 (*s*, CH–O–CH); 7.33–7.52 (*d*, *J* = 9.0, Ph); 7.53–7.67 (*d*, *J* = 9.0, Ph). ^{13}C-NMR (75 MHz, (D_6) DMSO): 28.43 ($2CH_2$); 33.72 ($CH_{2thiazol}$); 48.80 (2CH); 78.82 (CH–O–CH); 129.69 (2 *o*-PhCH); 130.38 (2 *m*-PhCH); 133.49 (*p*-PhC–Cl); 133.96 (*i*-PhC); 170.62 ($C_{thiazol}$); 171.80 (C=O); 172.69 (2C=O). HR-MS: 392.0125 ($[M+H]^+$, $C_{17}H_{14}ClN_3O_4S^+$; calc. 391.8288).

2-{[3-(4-Fluorophenyl)-4-oxo-1,3-thiazolidin-2-ylidene]amino}hexahydro-1*H*-4,7-epoxyisoindole-1,3(2*H*)-dione (**17**). Yield: 69%. M.p. 282–284 °C. ^{1}H-NMR (300 MHz, (D_6)DMSO): 1.65 (*s*, $2CH_2$); 3.11 (*s*, 2CH); 4.22 (*s*, CH_2); 4.80 (*s*, CH–O–CH); 7.27 – 7.56 (*m*, Ph). ^{13}C-NMR (75 MHz, (D_6)DMSO): 28.43 ($2CH_2$); 33.64 ($CH_{2thiazol}$); 48.80 (2CH); 78.82 (CH–O–CH); 116.43 (*m*-PhCH); 116.73 (*m*-PhCH); 130.81 (*o*-PhCH + *i*-PhC); 130.88 (*o*-PhCH); 160,49 (*p*-PhC–F); 163,97 (*p*-PhC–F); 170.78 ($C_{thiazol}$); 171.94 (C=O); 172.71 (2C=O). HR-MS: 376.0440 ($[M+H]^+$, $C_{17}H_{14}FN_3O_4S^+$; calc. 375.3742).

2-{[3-(2-Methylphenyl)-4-oxo-1,3-thiazolidin-2-ylidene]amino}hexahydro-1*H*-4,7-epoxyisoindole-1,3(2*H*)-dione (**18**). Yield: 72%. M.p. 292–294 °C. ^{1}H-NMR (300 MHz, (D_6)DMSO): 1.64 (*s*, $2CH_2$); 2.21 (*s*, Me); 3.09 (*s*, 2CH); 4.30 (*s*, CH_2); 4.68 (*s*, CH–O–CH); 7.26 – 7.41 (*m*, Ph). ^{13}C-NMR (75 MHz, (D_6)DMSO): 17.43 (Me); 28.43 ($2CH_2$); 33.62 ($CH_{2thiazol}$); 48.80 (2CH); 78.79 (CH–O–CH); 127.44 (*o*-PhCH); 129.02 (*p*-PhCH); 130.01 (*m*-PhCH); 131.29 (*m*-PhCH); 133.96 (*o*-PhC–Me); 136.44 (*i*-PhC); 169.85 ($C_{thiazol}$); 171.88 (C=O); 172.74 (2C=O). HR-MS: 372.0678 ($[M+H]^+$, $C_{18}H_{17}N_3O_4S^+$; calc. 371.4103).

2-[(3-Ethyl-4-oxo-1,3-thiazolidin-2-ylidene)-amino]hexahydro-1*H*-4,7-epoxyisoindole-1,3(2*H*)-dione (**19**). Yield: 76%. M.p. 182–184 °C. ^{1}H-NMR (300 MHz, (D_6)DMSO): 1.22 (*t*, *J* = 9.0, Me), 1.73 (*s*, $2CH_2$); 3.41 (*s*, 2CH); 3.81 (*q*, *J* = 9.0, CH_2Me); 4.18 (*s*, CH_2); 4.80 (*s*, CH–O–CH). ^{13}C-NMR (75 MHz, (D_6)DMSO): 12.28 (Me); 28.45 ($2CH_2$); 33.39 ($CH_{2thiazol}$); 38.32 (CH_2); 48.81 (2CH); 78.82 (CH–O–CH); 169.67 ($C_{thiazol}$); 172.11 (C=O); 172.86 (2C=O). HR-MS: 310.0609 ($[M+H]^+$, $C_{13}H_{15}N_3O_4S^+$; calc. 309.3409).

2-({3-[2-(Morpholin-4-yl)ethyl]-4-oxo-1,3-thiazolidin-2-ylidene}amino)hexahydro-1*H*-4,7-epoxyisoindole-1,3(2*H*)-dione (**20**). Yield: 83%. M.p. 180–182 °C. ^{1}H-NMR (300 MHz, (D_6)DMSO): 1.55 (*s*, $2CH_2$); 2.36 (*s*, CH_2–N–CH_2); 2.58 (*s*, CH_2); 3.13 (*s*, 2CH); 3.45 (*s*, CH_2–O–CH_2); 3.83 (*s*, CH_2); 4.14 (*s*, CH_2); 4.62 (*s*, CH–O–CH). ^{13}C-NMR (75 MHz, (D_6)DMSO): 28.46 (CH_2); 33.19 ($CH_{2thiazol}$); 40.05 (CH_2); 48.84 (2CH); 53.60 (CH_2–N–CH_2); 54.11 (CH_2); 66.64 (CH_2–O–CH_2); 78.77 (CH–O–CH); 169.95 ($C_{thiazol}$); 172.40(C=O); 172.83 (2C=O). HR-MS: 395.1036 ($[M+H]^+$, $C_{17}H_{22}N_4O_5S^+$; calc. 394.4454).

Antiradical Activity Analysis

Preliminary assessment of the antioxidant activity of synthesized compounds was screened using DPPH-TLC assay.[30,31] The solutions of analyzed compounds at concentration 0.033 M were developed on a silica gel TLC plate (Merck, Darmstadt, Germany) using: toluene/methanol/25% ammonia (9:1:0.05) solvent system. After drying, TLC plates were sprayed with 0.2% 1,1-diphenyl-2,2-picrylhydrazyl (DPPH, Fluka Co., Buchs, Switzerland) solution in methanol. Compounds showing a yellow-on-purple spot were regarded as antioxidants.

The antiradical activity was assayed using an improved $ABTS^{+\bullet}$ decolorization assay with some modifications.[32] ABTS was dissolved in water to a 8.7 mM concentration. ABTS radical cation ($ABTS^{+\bullet}$) was produced by reacting 1.25 ml of ABTS stock solution with 5 ml of potassium persulfate (12.2 mM) and allowing the mixture to stand in the dark at room temperature for 12–18 h before use. The $ABTS^{+\bullet}$ solution was diluted with methanol (1:100).

To determine EC_{50} of samples, the technique with 96-well microplates was used. Aliquots of 180 µl of a $ABTS^{+\bullet}$ solution were mixed with 20 µl of the samples diluted to various concentrations in 96-well microplates. Absorbance was recorded after 6 min of incubation at 734 nm using the Infinite Pro 200F microplate reader (Tecan Group Ltd., Männedorf, Switzerland). The ability of the extract to quench the $ABTS^{\bullet}$ free radicals was determined using the following equation:

$$\text{Scavenging } \% = [(\text{Ac}-\text{Aa})/\text{Ac}] \times 100$$

where Ac is the absorbance of control and Aa is the absorbance of sample.

Results of antioxidant activity were expressed as EC_{50} values [as μg sample per ml], defined as the amount of antioxidant necessary to decrease the initial $ABTS^{+\bullet}$ concentration by 50% and also as a Trolox equivalent antioxidant capacity (TEAC, [mM of Trolox per g sample]) based on their EC_{50} values.

Metal chelating power (CHEL) was determined by the method of *Guo et al.*[33] Subsequently, the absorbance of the solution was recorded spectrophotometrically at 562 nm. The percentage of inhibition of ferrozine-Fe^{2+} complex formation was calculated according to the following formula:

$$\% \text{ inhibition} = [1-(Ap/Ac)] \times 100$$

where Ac – the absorbance of control and Ap – the absorbance in the presence of sample.

Results were expressed as % inhibition of sample for concentration of 5 mg of sample per ml.

Author Contribution Statement

Anna Pachuta-Stec designed the study, performed the synthesis of new norcantharidin analogs, analyzed the spectral data and wrote the first draft of the manuscript excluding the antiradical activity analysis. Renata Nowak supervised the antiradical activity tests and wrote the antiradical activity section of this manuscript. Wioleta Pietrzak performed the antiradical activity analysis of synthesized compounds. Monika Pitucha has made revision of the article. All authors read and approved the final manuscript.

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