Abstract: One of the powerful antioxidants used clinically is Edaravone (EDA). We synthesized a series of new EDA analogs, 4-aminopyrazol-5-ol hydrochlorides, including polyfluoroalkyl derivatives, via the reduction of 4-hydroxyiminopyrazol-5-ones. The primary antioxidant activity of the compounds in comparison with EDA was investigated in vitro using ABTS, FRAP, and ORAC tests. In all tests, 4-Amino-3-pyrazol-5-ols were effective. The lead compound, 4-amino-3-methyl-1-phenylpyrazol-5-ol hydrochloride (APH), showed the following activities: ABTS, 0.93 TEAC; FRAP, 0.98 TE; and ORAC, 4.39 TE. APH and its NH-analog were not cytotoxic against cultured normal human fibroblasts even at 100 µM, in contrast to EDA. According to QM calculations, 4-aminopyrazolols were characterized by lower gaps, IP, and η compared to 4-hydroxyiminopyrazol-5-ones, consistent with their higher antioxidant activities in ABTS and FRAP tests, realized by the SET mechanism. The radical-scavenging action evaluated in the ORAC test occurred by the HAT mechanism through OH bond breaking in all compounds, directly dependent on the dissociation energy of the OH bond. All the studied compounds demonstrated the absence of anticholinesterase activity and moderate inhibition of CES by some 4-aminopyrazolols. Thus, the lead compound APH was found to be a good antioxidant with the potential to be developed as a novel therapeutic drug candidate in the treatment of diseases associated with oxidative stress.

Keywords: Edaravone; 4-aminopyrazol-5-ols; 4-hydroxyiminopyrazol-5-ones; antioxidants; antiradical and ferric reducing activity; quantum-chemical calculations

1. Introduction

Oxidative stress is a crucial factor in the development of serious pathological conditions including cancer, aging, atherosclerosis, rheumatoid arthritis, cardiovascular and autoimmune diseases, and neurodegenerative disorders. It is characterized by an imbalance between the formation and neutralization of free radicals arising from the dysfunction
of endogenous antioxidant protection systems. This redox abnormality leads to the critical accumulation of reactive oxygen and nitrogen species (ROS and RNS, respectively), such as peroxides and free radicals that damage cellular proteins, lipids, and nucleic acids. Antioxidants and radical scavengers play vital defensive roles by modulating the concentrations of ROS and RNS. Consequently, they can reduce the occurrence of disorders associated with oxidative stress [1,2].

The pyrazole core is considered to be a unique pharmacophore for the design of promising antioxidants [3–5]. One of the powerful antioxidants and neuroprotective drugs in clinical practice is 3-methyl-1-phenyl-2-pyrazolin-5-one (Edaravone, EDA, Radicut, Radicava). This prominent drug was originally used to treat brain infarction (stroke) [6–8] and was subsequently approved for the treatment of amyotrophic lateral sclerosis [9–11]. In addition, EDA can be used to treat Alzheimer’s disease, glaucoma, pulmonary diseases, cardiovascular dysfunctions, and medical chronic kidney damage, and to reduce specific side effects in the treatment of cancer [12–14]. Broadly speaking, EDA can be used to treat a range of diseases involving oxidative stress, and it has no severe adverse effects [12,15,16].

EDA has been found to be active in quenching free radicals, because the phenolic hydroxyl (exhibiting a propensity for scavenging free radicals) is generated from the tau-tomerization of its C=O group [17–21]. EDA effectively binds hydroxyl radicals by a consistent mechanism for electron–proton transfer [22,23] enabling it to react with alkoxyl radicals, superoxide anions, peroxyl radicals of lipids, peroxynitrite, and nitrogen(II) oxide [18,20,24]. EDA’s scavenging activity against multiple free radical species is as robust as other known potent antioxidants such as uric acid, glutathione, and Trolox [19].

Among analogs of EDA, 3-methyl-1-(pyridin-2-yl)-5-pyrazolone had the highest ability to scavenge radicals due to the increase in its active anion form stabilized by an intramolecular hydrogen bond in [25]. EDA derivatives bearing the NO-donor group in an aryl substituent showed different degrees of balance between antioxidant and vasodilating activity in vitro in [26,27]. Analogs having thiazolyl residues also demonstrated pronounced antioxidant properties in the ABTS test in [28]. Among 4-aryl- and 4-carboxyamide-MeO-derivatives of EDA, compounds with antioxidant properties in vitro were found in [29]. Furthermore, in another study, a series of DL-3-n-butyolphthalide-EDA hybrids were synthesized as novel dual inhibitors of β-amyloid aggregation and monoamine oxidases for potential application as Alzheimer’s disease therapeutics, and these conjugates also possessed high antioxidant activity [30].

Thus, the EDA pyrazolone structure can be considered as a promising scaffold for the design of new effective antioxidants. This encouraged us to create new 4-amino-substituted EDA analogs and study their antioxidant properties. We believe that pyrazoles combining amino and hydroxyl groups in their structures should be promising as antioxidants.

For the synthesis of the 4-amino-5-alkoxy-3-methyl-1-phenylpyrazole hydrochloride, the use of reduction of the 4-nitro group in 5-RO-pyrazoles under the action of SnCl₂ in concentrated HCl was described in [31]. Furthermore, 1-(sulfonated phenyl)-4-amino-5-pyrazolones were obtained through the reduction of hydroxyimino derivatives by zinc in a mixture of hydrochloric and acetic acids in [32] and by catalytic hydrogenation under the action of Pd/C in [33]. For the synthesis of N-glycoconjugated 4-amino-3-methyl-5-pyrazolones, the reaction was carried out with zinc in methanol in the presence of NH₄Cl in [34]. In addition, a method was proposed for the synthesis of 3-substituted 4-aminopyrazol-5-ol hydrochlorides via cyclization of 2-amino-3-oxo esters with hydrazine in [35]. However, it should be noted that although the 4-amino derivative of EDA and its NH-unsubstituted analog were obtained in this way, there was no information on their structures.

Data on polyfluoroalkyl-containing 4-aminopyrazolols are limited to two examples describing the synthesis of 4-alkylamino-3-trifluoromethylpyrazol-5-ols via the recyclization of the relatively inaccessible compound 4-trifluoroacetyl-1,3-oxazolium-5-olate [36] or 5-trifluoromethyl-4-ethoxy carbonyl-1,3-oxazole [35] under the action of hydrazines. However, fluorinated derivatives, including pyrazoles, are undoubtedly promising as
pharmacological agents due to the presence of fluorine atoms that change the physical, chemical, and biological properties of organic molecules [37–44]. For example, incorporation of fluorine atoms into drugs can increase their metabolic stability and lipophilicity, thereby facilitating penetration of the molecules through biological membranes [45].

Herein, we focused on the synthesis of 4-amino-substituted EDA derivatives, including polyfluoroalkyl-containing analogs, and the study of their primary antioxidant activity in comparison with EDA, CF$_3$EDA, and Trolox using ABTS, FRAP, and ORAC tests. To explain the factors influencing antioxidant activity, we performed quantum-chemical calculations. Taking into account the literature data on the ability of pyrazole derivatives to inhibit acetylcholinesterase [46–50] and human carboxylesterase-1 [51], we also investigated the esterase profiles of the new compounds, i.e., their inhibitory activity against acetylcholinesterase (AChE), butyrylcholinesterase (BChE), and carboxylesterase (CES). In addition, the effect of the compounds on cell viability was evaluated in normal human dermal fibroblast cultures.

2. Results and Discussion
2.1. Chemistry

For the synthesis of polyfluoroalkyl-containing 4-aminopyrazol-5-ols, we used the reduction of 4-hydroxyiminopyrazolones 1a–j, for which we have recently proposed convenient synthesis methods [52].

First, we tried to carry out reduction of 4-hydroxyiminopyrazolones 1a,b with zinc in acetic acid, according to our successful method for the synthesis of 4-amino-3-trifluoromethyl-5-alkyl[(het)aryl]pyrazoles from 4-nitrosopyrazoles [53]. However, this method did not allow us to obtain the expected 4-aminopyrazol-5-ols, because bis-[5-hydroxy-1-phenyl-3-(polyfluoroalkyl)-1H-pyrazol-4-yl]amines 2a,b were isolated from these reactions as a result of crosslinking of two pyrazolone molecules by the amino group (Scheme 1). Similar transformations have been described for non-fluorinated analogs [35,54]. Although the mechanism of formation of these compounds is unclear, it can be assumed that the free amines are unstable and convert to bis-pyrazoles 2a,b due to oxidation under the action of oxygen in the air [55].

![Scheme 1. Reduction of 4-hydroxyiminopyrazolones 1a-j.](image-url)
The bis-pyrazole structure of compound 2a was confirmed by XRD (Figure 1). In its crystal structure, the symmetry axis passing through the exocyclic nitrogen atom N3 and the proton H1 of the hydroxyl group can be marked. The two pyrazole molecules are mirror images of each other and generate a “crab”-like form. The eight-membered pseudocycle O1C5C4N3C4C5O1H1 is practically flat, with the O1 oxygen atom deviating from the plane by 0.353 Å. The compound is characterized by delocalization of double bonds (the lengths of C4N3 and N4C3 are equal to 1.312 Å), and the proton H1 of the hydroxyl group is delocalized between the two O1 oxygen atoms, with the distance O1H1 equal to H1O1 (1.208 Å).

![Figure 1. The structure of compound 2a according to XRD.](image-url)

When adding acetic anhydride to the reducing mixture Zn/AcOH, reduction of the hydroxylimine group of pyrazolone 1a was accompanied by acylation of the resulting amino function to yield 4-acylamino pyrazol-5-ol 3a (Scheme 1).

4-Aminopyrazol-5-ols 4a–c,f,i, as hydrochlorides, were synthesized by reducing 4-hydroxyiminopyrazolones 1a–c,f–i under the action of tin(II) chloride in concentrated HCl (Scheme 1). However, the use of this synthetic protocol was not effective for the preparation of NH-unsubstituted 4-amino-3-CF3-pyrazol-5-ol 4e, and catalytic hydrogenation in EtOH-HCl in the presence of Pd/C was used for its synthesis. The application of such a system facilitates the isolation of amine salts, as shown in the preparation of compound 4c.

The same method was used to prepare the 4-amino derivative of EDA 4d and its NH-unsubstituted analog 4j from the corresponding 4-hydroxyiminopyrazol-5-ones 1d,j by catalytic reduction (Scheme 1). Compounds 4d,j were characterized by IR along with 1H and 13C NMR spectroscopy, because spectral data for these compounds were lacking [35].

Attempts to carry out catalytic hydrogenation of compounds 1a,b,d under acid-free conditions led to bis-pyrazoles 2a,b or to the resinification of the reaction mass. It can be concluded that 4-aminopyrazol-5-ol as a free base is unstable.

The structure of 4-aminopyrazoles 4a–j was confirmed by IR and NMR spectroscopy. The NMR spectra of these compounds recorded in DMSO-d6 are characterized by revealing one hydroxyl form that was confirmed by the presence in the 13C NMR spectra of compounds 4 of carbon atom signals of –C-OH at δ 147–154 ppm. According to NMR spectroscopy data, aminopyrazole hydrochlorides 4a,b (form AH) standing in DMSO solutions were able to convert to ammonium salts (form AS) (Scheme 1). Thus, in the 1H NMR spectrum of hydrochlorides 4a,b, the proton signals of the amino group were broad low-field signals or absent due to deuterium exchange with solvent water, while the spectra after standing of a sample for 6–12 h contain triplet signals of protons of NH3+ groups of ammonium salts. In addition, the content of the AS form increases with
elongation of the polyfluoroalkyl substituent. The AH:AS ratio was 77:23 (for 4a) and 40:60 (for 4b). The 13C NMR spectra of compounds 4a,b also contained two sets of signals characterized by the multiplet broadening of the signals of carbon atoms for the ammonium salt and by singlet signals of the same carbons for the hydrochloride. However, compound 4c bearing a nonfluorobutyl residue existed in DMSO solution only as AS, while the methyl-containing analog 4d occurred as AH. All N-unsubstituted aminopyrazoles 4e–j were revealed to adopt only the AH form.

2.2. Evaluation of Antioxidant Potential of Pyrazoles 1 and 4

For studying the primary antioxidant activity of compounds 1 and 4, three different methods were selected: (1) the FRAP test, which exclusively evaluates the single electron transfer (SET) mechanism; (2) the ORAC-FL test, evaluating exclusively the hydrogen atom transfer (HAT) mechanism; and (3) the ABTS test, which reflects either HAT or SET mechanisms as well as their combination. For all methods, Trolox was used as the reference antioxidant: antioxidant activity of the compounds was presented relative to the activity of Trolox. Quercetin was used as a positive control. The data are presented in Table 1.

Table 1. Antioxidant activity of pyrazoles 1 and 4.

| No. | Compound | R1 | R2 | ABTS (n = 3; 1 h) | FRAP (n = 3; 1 h) | ORAC-FL (n = 3; 2 h) |
|-----|----------|----|----|------------------|------------------|---------------------|
|     |          |    |    | TEAC * (IC50, µM) | TE * (IC50, µM)  |                     |
|     |          |    |    | TE * (IC50, µM)  |                  |                     |
| 1a  | CF3      | Ph |    | 0.035 ± 0.002    | n.a.             | n.a.                |
| 1b  | C2F5     | Ph |    | 0.03 ± 0.002     | 0.06 ± 0.01      | n.a.                |
| 1c  | C4F9     | Ph |    | 0.04 ± 0.004     | 0.06 ± 0.01      | n.a.                |
| 1d  | Me       | Ph |    | 0.16 ± 0.02      | n.a.             | 1.07 ± 0.17         |
| 1e  | CF3      | H  |    | 0.23 ± 0.01      | 0.07 ± 0.01      | 0.98 ± 0.03         |
| 1f  | H(CF2)2  | H  |    | 0.15 ± 0.007     | n.a.             | n.d.                |
| 1g  | C2F5     | H  |    | 0.13 ± 0.04      | 0.13 ± 0.01      | 1.87 ± 0.04         |
| 1h  | C3F7     | H  |    | 0.19 ± 0.008     | n.a.             | 2.37 ± 0.09         |
| 1i  | C4F9     | H  |    | 0.2 ± 0.01       | n.a.             | 2.41 ± 0.08         |
| 1j  | Me       | H  |    | 0.2 ± 0.03       | 0.09 ± 0.01      | 2.85 ± 0.28         |
The ABTS assay is based on the direct quenching of the ABTS cation radical (ABTS•+) by antioxidants. The assay is carried out by spectrophotometric determination of a decrease in absorbance of a stable dark green ABTS•+ solution after its interaction with an antioxidant compound [56]. The measurements were performed as previously described in detail [57]. The results are expressed as TEAC values (Trolox equivalent antioxidant capacity) and IC50 values (compound concentration required for 50% reduction of the ABTS radical).

| No. | Compound | ABTS (n = 3; 1 h) | FRAP (n = 3; 1 h) | ORAC-FL (n = 3; 2 h) |
|-----|----------|------------------|------------------|---------------------|
|     |          | TEAC * (IC50, µM) ** | TE * | TE * (IC50, µM) ** |
| 4a  | CF3      | 0.75 ± 0.04 (27.2 ± 2.0) | 0.89 ± 0.05 | 3.49 ± 0.16 (8.8 ± 0.1) |
| 4b  | C2F5     | 0.6 ± 0.03 (34.1 ± 2.0) | 0.75 ± 0.07 | 2.12 ± 0.17 |
| 4c  | C4F9     | 0.85 ± 0.04 (23.4 ± 2.1) | 0.98 ± 0.05 | 2.82 ± 0.2 |
| 4d  | Me       | 0.93 ± 0.03 (23.3 ± 1.9) | 0.98 ± 0.08 | 4.39 ± 0.04 (6.85 ± 0.7) |
| 4e  | CF3      | 0.92 ± 0.04 (21.2 ± 1.8) | 1.02 ± 0.02 | 0.90 ± 0.19 |
| 4f  | H(CF2)2  | 0.50 ± 0.02 (33.2 ± 2.3) | 0.62 ± 0.01 | n.d. |
| 4g  | C2F5     | 0.73 ± 0.03 (25.7 ± 2.1) | 0.88 ± 0.04 | 0.54 ± 0.03 |
| 4h  | C3F7     | 0.78 ± 0.04 (24.8 ± 1.6) | 1.03 ± 0.04 | 0.63 ± 0.04 |
| 4i  | C4F9     | 0.45 ± 0.02 (40.8 ± 1.1) | 0.75 ± 0.05 | 1.83 ± 0.02 |
| 4j  | Me       | 0.95 ± 0.04 (20.1 ± 1.8) | 1.04 ± 0.04 | 2.06 ± 0.16 |
| CF3-EDA |         | 0.95 ± 0.04 (21.4 ± 0.8) | 0.63 ± 0.03 | 3.89 ± 0.04 (6.3 ± 0.2) |
| EDA  |         | 0.96 ± 0.04 (21.4 ± 1.1) | 0.80 ± 0.01 | 3.71 ± 0.06 (5.6 ± 0.1) |
| Trolox     |         | 1.0 (20.1 ± 1.2) | 1.0 | 1.0 (23.6 ± 4.4) |
| Quercetin  |         | 1.20 ± 0.11 (13.8 ± 0.7) | 4.99 ± 0.02 | 5.41 ± 0.08 |

n.d.—not determined; n.a.—not active; * TEAC (Trolox equivalent antioxidant capacity, ABTS) and TE (Trolox equivalent, FRAP, ORAC)—activity of the compounds relative to Trolox (for calculation, see Experimental). ** compound concentration required for 50% reduction of the ABTS radical-cation or peroxyl radical.
The FRAP (ferric reducing antioxidant power) assay measures the ability of antioxidants to reduce the ferric 2,4,6-tripyridyl-s-triazine complex \([\text{Fe(TPTZ)}_2]^{3+}\) to the intensely blue ferrous complex \([\text{Fe(TPTZ)}_2]^{2+}\) in an acidic medium [58]. The measurements were performed as previously described in detail [59]. The results are expressed as TE (Trolox equivalents).

In the oxygen radical absorbance capacity (ORAC-FL) assay, 2,2′-azobis-(2-methylpropionamidine) dihydrochloride (AAPH) is used as a free peroxyl radical generator, and fluorescein (FL) is used as a fluorescent probe [60]. The method is based on measuring the decrease in the intensity of fluorescence with time, which characterizes the degree of decay of the fluorescent probe under the influence of peroxyl radicals. In the presence of antioxidants, the degree of decay of the fluorescent probe decreases and, accordingly, the fluorescence intensity increases. The results are expressed as TE (Trolox equivalents).

2.2.1. ABTS Assay

As shown in Table 1, fluorine-containing NH-unsubstituted 4-hydroxyiminopyrazol-5-ones 1e–i demonstrated moderate radical-binding activity in the ABTS test, which was practically independent of the R\textsubscript{F} substituent structure (TEAC = 0.13–0.23). The introduction of a phenyl substituent into position 1 of the pyrazole ring resulted in a sharp decrease in the antiradical activity of compounds 1a–c regardless of the R\textsubscript{F} structure: 1e vs. 1a, (R\textsubscript{F} = CF\textsubscript{3}); 1g vs. 1b, 1i vs. 1c (R\textsubscript{F} = C\textsubscript{4}F\textsubscript{9}).

The replacement of the fluorine-containing radical with the methyl group at position 3 promoted an increase in the radical-binding activity of the phenyl-containing derivative (1d vs. 1a) and did not affect the activity of the NH-unsubstituted derivative (1j vs. 1e). The latter compounds exhibited an equally moderate ABTS\textsuperscript{**}-binding activity.

In contrast to 4-hydroxyiminopyrazol-5-ones 1, aminopyrazoles 4 showed high radical-binding activity in the ABTS test at the level of Trolox and EDA. The ABTS-binding activity of 4-unsubstituted 3-trifluoromethyl-1-phenylpyrazol-5-ol (CF\textsubscript{3}-EDA) was found to be equal to that of EDA.

In addition, the introduction of a Ph substituent to position 1 of the pyrazole ring in 4-amino-3-R\textsubscript{F}-pyrazolols 4 retained their high antioxidant activity.

The elongation of the polyfluoroalkyl radical in NH-unsubstituted 4-aminopyrazoles 4 resulted in a gradual decrease in the ABTS-binding activity in the following order: 4e > 4g ~ 4h > 4i. The activity was halved in the transition from CF\textsubscript{3}-pyrazole 4e to the C\textsubscript{4}F\textsubscript{9} analog 4i.

Similarly to 4-hydroxyiminopyrazolones 1e,j, the replacement of the CF\textsubscript{3} group by a Me residue at position 3 fostered an increase in the ABTS\textsuperscript{**}-binding activity of aminopyrazol 4d in the presence of a Ph substituent at position 1 (4d vs. 4a) and retention of high activity in the case of NH-unsubstituted derivatives (4j vs. 4e).

Thus, aminopyrazoles 4c,d,e,j exhibited the greatest activity in the ABTS test and had TEAC values at the level of Trolox, EDA, and CF\textsubscript{3}-EDA. It is also noteworthy that our ABTS test data on the antioxidant activity of EDA matched the values reported in the literature [61,62].

2.2.2. FRAP Assay

Compounds 1 and 4 demonstrated structure–activity relationships in the FRAP assay close to the results from the ABTS test, although in some cases they were less pronounced.

Thus, 4-hydroxyiminopyrazolones 1 were either inactive or exhibited very low activity compared to the standard antioxidant Trolox. The introduction of a phenyl substituent into position N1 reduced ferric reducing activity and, correspondingly, antioxidant activity: 1a vs. 1e (R = CF\textsubscript{3}), 1b vs. 1g (R = C\textsubscript{2}F\textsubscript{5}), 1d vs. 1j (R = Me).

As in the ABTS test, 4-aminopyrazolols 4 were significantly more active as antioxidants compared to 4-hydroxyiminopyrazolones 1. As shown in Table 1, most of the compounds of this group showed high activity at the level of the standard antioxidant Trolox.
The introduction of a Ph-substituent at position 1 of the pyrazole ring of compounds 4 retained their high antioxidant activity compared to unsubstituted ones. Activity increased for compounds with $R^F = C_6F_5$ (4c was more active than 4i).

Similar to the results of the ABTS test, the replacement of the CF$_3$ group with CH$_3$ at position 3 promoted an increase in ferric reducing activity in the presence of a Ph substituent at position 1 (4a vs. 4d) and maintained high activity in the case of the NH-unsubstituted derivatives (4e vs. 4j).

Interestingly, according to literature data [25], CF$_3$-EDA was 3 times less active compared to EDA in the OH radical binding test (hydroxyl radical scavenging activity). Meanwhile, in the ABTS test, the activity of CF$_3$-EDA was at the level of EDA and Trolox, and in the FRAP test, its ferric reducing activity was only 20% lower than that of EDA (Table 1).

Thus, Ph-substituted (4a,c,d) and NH-unsubstituted (4e,g,h,j) 4-aminopyrazoles showed high activity in the FRAP test. In addition, compounds 4c,d,e,j showed significant activity in the ABTS test. Moreover, the ferric reducing activity of compounds 4a,c,d,e,g,h,j was at the level of Trolox and exceeded the activity of EDA or CF$_3$-EDA.

2.2.3. ORAC Assay

The ORAC assay utilizes a biologically relevant radical source, 2,2′-azobis(2-aminopropyl) dihydrochloride (AAPH)-derived peroxyl radicals. Thus, it is a more physiologically relevant assay than ABTS and FRAP.

As shown in Table 1, overall, the results of the ABTS and FRAP tests agree with the ORAC-FL data; i.e., all compounds that were active in the first two tests showed a pronounced antioxidant effect in the ORAC test. However, there were also differences. In particular, the methyl analog 1d showed activity at the Trolox level (TE = 1.07) in the ORAC test. In addition, NH-unsubstituted 4-hydroxyiminopyrazol-5-ones 1e,h,i,j, which were inactive or low-active in the FRAP test or exhibited moderate activity in the ABTS test, showed a pronounced radical binding effect in the ORAC test. This effect was higher for compounds with a longer polyfluoroalkyl substituent (1e < 1h = 1i).

Structure–activity analysis for results in the ORAC test showed that all compounds in the series of 4-amino derivatives 4 exhibited high antioxidant activity, in contrast to 4-hydroxyiminomino derivatives 1. The introduction of an N-phenyl substituent into position 1 significantly enhanced the effect (4a vs. 4e, 4b vs. 4g, 4c vs. 4i, 4d vs. 4j).

In each of the subgroups 1a–d, 1e–j, and 4a–d, 4e–j, compounds 1d,j and 4d containing a methyl group instead of a polyfluoroalkyl group exhibited higher activity. The most active among the aminopyrazolols 4 were compounds 4a (R$^1 = CF_3$, R$^2 = Ph$, 3.49 TE) and 4d (R$^1 = Me$, R$^2 = Ph$, 4.39 TE).

In particular, the lead compound 4d was more active than EDA (3.71 TE) and CF$_3$-EDA (3.89 TE).

IC$_{50}$ values were determined for compounds 4a,d,e, EDA, CF$_3$-EDA, and Trolox (Table 1). The obtained IC$_{50}$ values were 3–5 times lower than the IC$_{50}$ of Trolox, again confirming the high antioxidant activity of the tested pyrazole derivatives.

Thus, the results of the ABTS and FRAP tests show that 4-hydroxyiminopyrazolones 1 had moderate or very weak activity (HN-derivatives 1e–j were more active than Ph-N-analogs 1a–d), while 4-aminopyrazolols 4 demonstrated a pronounced antioxidant effect comparable to Trolox (additionally, HN- and PhN-derivatives 4a–d and 4e–j had close activity). The most active compounds in both of these tests were the 4-aminopyrazolols 4d,e,j. In contrast, HN-unsubstituted 4-hydroxyiminopyrazolones 1e–j and all 4-aminopyrazolols 4a–j were active in the ORAC test. The increase in activity of 4-aminopyrazolol 4 was promoted by the introduction of a phenyl substituent and the replacement of a fluoroalkyl substituent with a methyl group. The lead compound 4d was more active in all tests than EDA and CF$_3$-EDA.
2.3. Assessment of Esterase Profile of Pyrazoles 1a–j and 4a–j

Given the available literature data on the ability of pyrazole derivatives to inhibit acetylcholinesterase (AChE) [46–50] and human carboxylesterase-1 (CES) [51], we performed an investigation of the esterase profile for new compounds 1a–j and 4a–j, including an evaluation of their inhibitory effect on the standard enzymes set: human erythrocyte AChE, equine serum butyrylcholinesterase (BChE), and porcine liver CES (Table 2). The applicability of this set of enzymes was shown by us earlier [63–66].

Table 2. Esterase profile of pyrazoles 1 and 4.

| No. | Compound | AChE | BChE | CES |
|-----|----------|------|------|-----|
|     | R¹ | R² |       | % Inhibition at 20 μM ¹ or IC₅₀, μM ² |     |
| 1a  | CF₃ | Ph  | n.a. ³ | n.a. | n.a. |
| 1b  | C₂F₅ | Ph  | 11.9 ± 0.9% | 16.1 ± 1.4% | 8.2 ± 1.3% |
| 1c  | C₄F₉ | Ph  | 11.5 ± 1.8% | 5.3 ± 1.0% | 7.8 ± 1.4% |
| 1d  | Me  | Ph  | 4.9 ± 1.3%  | 6.6 ± 1.3% | n.a.  |
| 1e  | CF₃ | H   | n.a.  | n.a.  | 5.9 ± 1.1% |
| 1f  | H(CF₂)₂ | H  | 6.8 ± 1.2% | 4.7 ± 1.0% | 3.5 ± 0.9% |
| 1g  | C₂F₅ | H   | n.a.  | n.a.  | 7.8 ± 1.4% |
| 1h  | C₃F₇ | H   | 5.3 ± 1.3% | n.a.  | 6.6 ± 1.4% |
| 1i  | C₄F₉ | H   | n.a.  | n.a.  | n.a.  |
| 1j  | Me  | H   | 3.8 ± 1.3% | n.a.  | n.a.  |

| 4a  | CF₃ | Ph  | 7.9 ± 1.5% | 6.9 ± 1.1% | 44.9 ± 4.0 |
| 4b  | C₂F₅ | Ph  | 4.4 ± 1.1% | 12.8 ± 1.5% | 16.6 ± 1.4% |
| 4c  | C₄F₉ | Ph  | 19.2 ± 1.7% | 10.3 ± 1.2% | 10.2 ± 0.8 |
| 4d  | Me  | Ph  | 6.5 ± 1.2%  | 5.1 ± 1.3% | 98.7 ± 8.8 |
| 4e  | CF₃ | H   | 9.7 ± 1.5%  | 5.6 ± 1.5% | n.a.  |
| 4f  | H(CF₂)₂ | H  | 17.3 ± 1.5% | n.a.  | 17.5 ± 1.4% |
| 4g  | C₂F₅ | H   | 17.3 ± 1.7% | 8.9 ± 1.4% | 6.7 ± 1.2% |
| 4h  | C₃F₇ | H   | n.a.  | 3.4 ± 0.9% | 81.8 ± 7.3 |
| 4i  | C₄F₉ | H   | 5.1 ± 0.9%  | n.a.  | 21.8 ± 1.7 |
| 4j  | Me  | H   | 13.9 ± 1.6% | n.a.  | 4.3 ± 0.9% |

| CF₃-EDA |  | n.a. | n.a. | 7.6 ± 1.2% |
| EDA     |  | n.a. | 4.6 ± 0.2% | 8.4 ± 0.7% |

¹ Percentages correspond to percent inhibition ± SEM at 20 μM, n = 3. ² Values without units of measurement (numbers printed in bold) correspond to IC₅₀ ± SEM in μM, n = 3. ³ n.a.—not active.

It was found that 4-hydroxyminopyrazolones 1a–j did not substantially inhibit any of the esterases, while aminopyrazolols 4a, c, d, h, i showed moderate anticarboxylesterase activity with IC₅₀ values in the range 10–98 μM, with a maximum activity of 10 and 22 μM for compounds 4c and 4i, respectively, which had a nonfluororobutyl substituent. These data indicate the absence of anticholinesterase side effects, with the potential therapeutic
application of these compounds and some probability of undesirable drug interactions from the use of aminopyrazolols 4 at high doses, owing to the inhibition of CES, which participates in the primary metabolism of numerous drugs with ester groups.

2.4. Quantum-Chemical Calculations of Antioxidant Activity

Herein, we used three analysis methods based on different reaction mechanisms, namely ORAC (HAT), FRAP (SET), and ABTS (SET or/and HAT), for an experimental assessment of antioxidant activity (AOA) of analogs of EDA 1a–j, 4a–j [67,68]. We noted that the values obtained with the ABTS and FRAP assays were significantly correlated (\(\rho = 0.702, p = 0.003\)), which was not the case for ABTS and ORAC (\(\rho = 0.391, p = 0.134\)) or FRAP and ORAC (\(\rho = 0.006, p = 0.985\)) (Spearman non-parametric correlation). These results may indicate the same SET mechanism of the antioxidant action of compounds 1a–j, 4a–j in FRAP and ABTS tests, whereas another mechanism (HAT) is realized in ORAC assay.

Computational strategies are widely used to investigate AOA [69–73]. To identify parameters responsible for the antioxidant effect of the studied compounds 1a–j and 4a–j, we performed theoretical calculations of their electronic characteristics using the GAUSSIAN 09 program [74]. The most popular hybrid functional (B3LYP) [75] combined with the basis sets cc-pVDZ [76] was used throughout the work for geometry optimization and frequency calculations. Moreover, geometrical parameters of 4a–j were optimized using the Polarizable Continuum Model (PCM) [77].

The scavenging activity of the aromatic compounds was shown to be directly proportional to the negative of the gaps computed as the energy difference between the HOMO and LUMO [78]; thus, we calculated such values for the synthesized analogs EDA 1a–j and 4a–j (Table 3).

### Table 3. Vertical values (IP, EA, \(\eta\)) and BDE(OH) of compounds 1a–j, 4a–j.

| R\(^1\) | \(\text{CF}_3\) | \(\text{C}_2\text{F}_5\) | \(\text{C}_4\text{F}_9\) | Me | \(\text{CF}_3\) | \(\text{(CF}_2\text{)}_2\text{H}\) | \(\text{C}_2\text{F}_5\) | \(\text{C}_3\text{F}_7\) | \(\text{C}_4\text{F}_9\) | Me |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Compounds | 1a | 1b | 1c | 1d | 1e | 1f | 1g | 1h | 1i | 1j |
| IP, eV | 8.45 | 8.44 | 8.44 | 7.98 | 9.84 | 9.61 | 9.78 | 9.76 | 9.77 | 9.10 |
| EA, eV | 1.49 | 1.55 | 1.60 | 0.97 | 1.21 | 1.11 | 1.28 | 1.31 | 1.34 | 0.58 |
| | (gap), eV | 6.96 | 6.89 | 6.84 | 7.01 | 8.63 | 8.5 | 8.5 | 8.45 | 8.43 | 8.52 |
| \(\eta\), eV | 3.48 | 3.45 | 3.42 | 3.50 | 4.32 | 4.25 | 4.25 | 4.22 | 4.21 | 4.26 |
| BDE (OH), kJ/mol | 315.4 | 314.3 | 314.3 | 314.0 | 314.0 | 320.7 | 313.0 | 312.9 | 313.0 | 312.7 |
| Compounds | 4a | 4b | 4c | 4d | 4e | 4f | 4g | 4h | 4i | 4j |
| IP, eV | 6.81 | 6.85 | 6.84 | 6.61 | 7.06 | 7.02 | 7.09 | 7.13 | 7.11 | 6.56 |
| EA, eV | 1.33 | 1.34 | 1.40 | 1.05 | 0.48 | 0.46 | 0.59 | 0.43 | 0.53 | 0.12 |
| | (gap), eV | −5.47 | −5.51 | −5.44 | −5.36 | −6.58 | −6.56 | −6.50 | −6.67 | −6.58 | −6.43 |
| \(\eta\), eV | 2.74 | 2.75 | 2.72 | 2.68 | 3.29 | 3.28 | 3.25 | 3.33 | 3.29 | 3.22 |
| BDE (OH), kJ/mol | 318.9 | 319.9 | 319.4 | 302.3 | 320.1 | 320.8 | 330.7 | 330.2 | 329.8 | 309.2 |

\(EA\)—electron affinity, \(IP\)—ionization potential, HOMO—the highest occupied molecular orbital, LUMO—the lowest unoccupied molecular orbital; \(\eta\)—chemical hardness, BDE—bond dissociation energy.

As is known, the ionization potential \(IP\) is directly related to SET reactions; such an approach is valuable and provides primary physicochemical insight into the mechanism of action in tests where the SET mechanism is operational. Therefore, we analyzed the relationships of the AOA of compounds 1a–j, 4a–j defined in the ABTS and FRAP tests with their structure, using indices calculated from the vertical values of \(IP\) and \(EA\). The use of only vertical values is due to the fact that the loss or capture of an electron by a molecule leads to an essential change in the structure and, consequently, the composition and energies...
of molecular orbitals. The vertical IP and EA quantities of all studied compounds 1a–j, 4a–j were collected in Table 3.

According to the experimental results, compounds 1 and 4 differed significantly in activity (Table 1), and the IP and gap calculation data confirmed this. For correct interpretation of the calculated quantum-chemical results, it is reasonable to consider the properties of compounds 1 and 4 within the following subgroups: polyfluoroalkyl (1a–c and 4a–c) and methyl (1d and 4d) derivatives with a phenyl substituent and polyfluoroalkyl (1e–f and 4e–i) and methyl (1j and 4j) derivatives with an NH fragment.

According to the data in Table 3, 4-aminopyrazolols 4a–j have lower IP, gap, and η values compared to 4-hydroxyiminopyrazolones 1a–j. This suggests that compounds 4a–j should be more active than pyrazolones 1a–j, and this was observed experimentally.

Compounds 1a–d containing the N-Ph moiety have lower IP, gap, and η values compared to NH analogs 1e–j. However, according to experimental data (Table 1), 4-hydroxyiminopyrazolones 1a–d having a N-Ph group exhibited less antioxidant activity compared to HN-unsubstituted derivatives 1e–j. Polyfluoroalkyl-containing compounds 1a–c and 1e–i have slightly higher IP values than methyl derivatives 1d and 1j. In this case, their gap and η values were nearly equal. According to the experiment, the fluorine-containing derivatives were somewhat less active than their methyl analogs.

4-Aminopyrazolol hydrochlorides 4 exhibited a pronounced antioxidant activity compared to 4-hydroxyiminopyrazolones 1 (Table 1), and this is consistent with lower IP, gap, and η values of compounds 4a–j compared to 4-hydroxyiminopyrazolones 1a–j. In the series of compounds 4, the property-changing tendency was similar to compounds 1 as a whole: compounds 4a–d containing the N-phenyl moiety had lower IP, gap, and η values compared to compounds 4e–j. However, the activities of both sets 4a–d and 4e–j in the antioxidant experiment were approximately the same. Methyl derivatives 4d and 4j had a more pronounced antioxidant activity, and this was consistent with their lower IP, gap, and η values than those of fluorinated analogs 4a and 4e.

Next, we analyzed the dependencies of AOA (TEAC) on the calculated parameters of IP, gap, and η (eV) for compounds 1 and 4 according to the work of Horton et al. [78] for aromatic compounds having hydroxyl and amino groups.

Considering the possible dependencies of the experimentally found AOA values in the ABTS test (as more complete) on the calculated IP, gap, and η values for 4-hydroxyiminopyrazolones 1a–j, we found that there was no direct dependence between the calculated IP and gap parameters with experimental AOA values.

At the same time, in the series of 4-aminopyrazolols 4 for all PhN-substituted compounds 4a–d, there were good negative dependences between their AOA values and gap functions (Figure 2a). It was not possible to identify acceptable relationships for HN-unsubstituted analogs 4e–j, because derivatives 4j and 4f containing Me- and H(CF2)2-substituents were expected to fall out of this series. For aminopyrazoles 4e.g.h–i containing perfluoroalkyl substituent F(CF2)h, there was a dependence of their AOA on the IP function, with a favorable coefficient of determination (Figure 2b).

According to the lower gap values obtained for compounds 1a–d and partially for aminopyrazoles 4a–d with phenyl substituents, a higher AOA could be expected compared to HN-heterocycles 1e–j and 4e–j. However, this effect was observed for only one pair of 4-aminopyrazolols, 4c and 4i. Differences in theoretical and experimental data can be explained by the structure of these polyfunctional compounds and various transformations of molecules with a PhN substituent and HN group during antioxidant action. In addition, it is possible that the ABTS-binding effect of compounds 1 and 4 is realized not only by the SET mechanism but also partially by the HAT mechanism. This is supported by an incomplete correlation of the values from the ABTS test with the antioxidant action values from the FRAP test, where only the SET mechanism is implemented (Table 1).
(Schemes 2 and 3), we calculated the BDE values for -OH bond breaking in compounds presented in Scheme 2.

For 4-aminopyrazolols 4a–d, similar transformations can also be assumed, but taking into account the reactions of the hydroxyl and the amino groups, this leads to more complex and varied conversions. Thus, CF$_3$-containing 4-aminopyrazolols 4a,e can theoretically form two J$_1$, J$_2$ (for 4a) or three K$_1$–K$_3$ (for 4e) radicals (Scheme 3). Non-fluorinated analogs 4d,j can undergo reactions involving a Me group, thereby forming three L$_1$–L$_3$ (for 4d) or four M$_1$–M$_4$ (for 4j) radicals. Evaluating the difference between the calculated BDE values, the most likely process for compounds 4a,d,e,j was found to generate radicals J$_1$, K$_1$, L$_1$, and M$_1$, forming via -O-H bond breaking (the ABDE values are indicated in blue in Scheme 3) despite the position of this substituent in these pyrazoles changes. This process is similar to the transformations of structures 1a,d,e,j.

Given that by using quantum-chemical calculations for pyrazoles 1d,j, 4d,j, it was shown that the formation of O-radicals is energetically most advantageous in all cases (Schemes 2 and 3), we calculated the BDE values for -OH bond breaking in compounds 1a–j and 4a–j (Table 3). This revealed a linear relationship with a highly favorable coefficient of determination between the AOA from the ORAC test and the BDE (OH) values for a series of active NH-unsubstituted 4-hydroxyiminopyrazolones 1e–j (Figure 3a).

Figure 2. Antioxidant activity (АОА, ABTS) vs. gap functions for 4-aminopyrazolols 4a–d (a) and (АОА, ABTS) vs. IP functions for 4-aminopyrazolols 4e, 4h–i (b).
Given that by using quantum-chemical calculations for pyrazoles \(1d, j, 4d, j\), it was shown that the formation of O-radicals is energetically most advantageous in all cases (Schemes 2 and 3), we calculated the BDE values for -OH bond breaking in compounds \(1a-j\) and \(4a-j\) (Table 3). This revealed a linear relationship with a highly favorable coefficient of determination between the AOA from the ORAC test and the BDE (OH) values for a series of active NH-unsaturated 4-hydroxyiminopyrazolones \(1e-j\) (Figure 3a).

Scheme 2. The formation of possible radicals in the reactions of 2-hydroxyiminopyrazolones \(1a, d, e, j\) with values of the difference in bond dissociation energies (\(\Delta BDE, \text{kJ/mol}\)), which were evaluated in the approximation of the gas phase (B3LYP/cc-pVDZ method).

Scheme 3. The formation of possible radicals in the reactions of 4-aminopyrazolols \(4a, d, e, j\) \(^*\) \((* \text{ conversions are given for 4-aminopyrazolols})\), with values of the difference in bond dissociation energies (\(\Delta BDE, \text{kJ/mol}\)), which were evaluated in the approximation of the gas phase (B3LYP/cc-pVDZ method).
Scheme 3. The formation of possible radicals in the reactions of 4-aminopyrazolols \(4a\),\(d\),\(e\),\(j\) (\(\star\)) (conversions are given for 4-aminopyrazolols), with values of the difference in bond dissociation energies (\(\Delta\)BDE, kJ/mol), which were evaluated in the approximation of the gas phase (B3LYP/cc-pVDZ method).

Figure 3. Antioxidant activity (AOA, ORAC) vs. BDE functions for 4-hydroxyimino-1-phenylpyrazolones \(1e\)–\(j\) (a) and antioxidant activity (AOA, ORAC) vs. BDE functions for polyfluoroalkyl 4-aminopyrazolols \(4a\)–\(c\), \(4e\)–\(i\) (b).

All tested aminopyrazolols \(4a\)–\(e\),\(g\)–\(j\) were active in the ORAC test, and an acceptable linear relationship between AOA and BDE for polyfluoroalkyl derivatives of \(4a\)–\(c\),\(e\),\(g\)–\(i\), was found (Figure 3b), regardless of the presence of a Ph substituent at the nitrogen atom in the molecule. It was impossible to find such a pattern for non-fluorinated analogs owing to the limited samples including only \(4d\), \(4j\).

According to the calculated BDE values (Table 3), HN-unsubstituted 4-hydroxyiminopyrazolones \(1e\)–\(j\) are more active than 4-aminopyrazolols \(4e\)–\(j\), and this is consistent with the AOA values from the ORAC test. According to the BDE values, the PhN-containing 4-aminopyrazolols \(4a\)–\(d\) should be more active than the HN analogs \(4e\)–\(j\), which also coincides with the experiment.

2.5. Cytotoxicity Studies

To investigate the effect of the lead compound \(4d\) as well as its NH analog \(4j\) in comparison with EDA on cell viability, cytotoxicity studies for these compounds were performed on normal human dermal fibroblast (NHDF) cultures using the MTT/formazan assay [81].

EDA appeared to have a dose-dependent effect on NHDF culture viability, the inhibitory capability of which increased with rising concentration (Table 4).

| EDA Inhibitory Capability (IC, %) at Increasing Concentrations | 1 \(\mu\)M | 10 \(\mu\)M | 100 \(\mu\)M |
|---------------------------------------------------------------|-----------|-----------|-----------|
| 3.74 ± 0.05                                                   | 19.9 ± 0.8| 32.8 ± 0.6|

Meanwhile, at a concentration of 100 \(\mu\)M, neither of the compounds \(4d,j\) showed cytotoxicity against NHDF cell culture.

3. Materials and Methods

3.1. Chemistry

Acetic acid, chloroform, dimethylsulfoxide, ethanol, \(n\)-hexane, hydrochloric acid, and zinc (dust) were obtained from AO “VEKTON” (St. Petersburg, Russia). Pd/C (5 wt.%) was purchased from Alfa Aesar by Thermo Fisher Scientific (Kandel, Germany). The deuterosolvent DMSO-\(d_6\) was acquired from “SOLVEX” Limited Liability Company (Skolkovo...
3.1.1. Synthesis of Rubazonic Acids 2a,b (General Procedure)

Pyrazolone 1a,b (5 mmol) was dissolved in glacial acetic acid (5 mL), and zinc dust (0.98 g, 15 mmol) was added. The reaction mixture was stirred for 4 h at room temperature (r.t.) and left standing overnight. Then, water (20 mL) was added, and the precipitate was filtered off and dried.

Bis[5-hydroxy-1-phenyl-3-(trifluoromethyl)-1H-pyrazol-4-yl]imine (2a). Yield 1.33 g (57%), red crystals, m.p. 188–189 °C. 1H NMR (500 MHz, CDCl3): δ 7.40–7.43, 7.50–7.53, 7.85–7.87 (all m, 10H, 2Ph); 17.16 (s, 1H, OH). 13C NMR (500 MHz, CDCl3): δ 119.23 (q, CF3, J 272.2 Hz); 121.29; 123.24; 128.33; 129.29; 136.42; 144.39 (q, C–CF3, J 37.4 Hz); 151.96. 19F NMR (500 MHz, CDCl3): δ 96.93 (s, CF3). Anal. calcd. for C20H11F6N2O2: C 51.40; H 2.37; N 14.94. Found: C 51.54; H 2.32; N 14.94.

Crystallographic data for compound 2a. The X-ray studies were performed on an “Xcalibur 3 CCD” diffractometer with a graphite monochromator, ω scanning with 1° step, λ(MoKα) 0.71073 Å radiation, T 295(2) K. Empirical absorption correction was applied. Using Olex2 [82], the structure was solved with the ShelXS [83] structure solution program using Direct Methods and refined with the ShelXL [84] refinement package using Least Squares minimization. All non-hydrogen atoms were refined in the anisotropic approximation; H-atoms at the C–H bonds were refined in the “rider” model with dependent displacement parameters. Empirical absorption correction was carried out through spherical harmonics, implemented in SCALE3 ABSPACK scaling algorithm by the program “CrysalisPro 1.171.36.32” (Rigaku Oxford Diffraction, 2013).

Suitable red single crystals of compound 2a were obtained by slow crystallization from CHCl3. Main crystallographic data for 2a: C20H11F6N2O2, M = 467.34, space group C2/c, monoclinic, a 19.559(2), b 20.5241(19), c 9.6419(16) Å; β 93.650(12)°; V = 3862.8(8) Å3; Z 8; μ 0.147 mm−1; 303 refinement parameters; 11207 reflections measured; 3921 unique (Rint 0.0664), which were used in all calculations. The final χ2 was 0.1299 (all data), and R1 was 0.0588 [I >= 2σ (I)]. CCDC 2183294 contains the supplementary crystallographic data for this compound.

Bis[5-hydroxy-1-(pentafluoroethyl)-1H-pyrazol-4-yl]imine (2b). Yield 1.28 g (45%), red crystals, m.p. 134–135 °C. 1H NMR (500 MHz, CDCl3): δ 7.40–7.43, 7.50–7.54, 7.86–7.88 (all m, 10H, 2Ph); 16.96 (s, 1H, OH). 19F NMR (CDCl3): δ 46.75 (br, s, 2F, CF2); 78.71 (br, s, 3F, CF3). Anal. calcd. for C22H11F10N2O2: C, 46.58; H, 1.95; N, 12.34. Found: C, 46.46; H, 2.04; N, 12.21.

N-(5-Hydroxy-3-(trifluoromethyl)-1-phenyl-1H-pyrazol-4-yl)acetamide (3a). Pyrazolone 1a (0.51 g, 2 mmol) was dissolved in glacial acetic acid (5 mL), and zinc dust (0.25 g, 4 mmol) was added. The reaction mixture was stirred for 30 min at r.t. Then, acetic anhydride (1 mL) was slowly added at 0 °C, and the reaction was left standing overnight at r.t. Then, water (20 mL) was added, and the precipitate was filtered off. The residue was purified by
column chromatography (eluent-CHCl₃/EtOH-10:1). Yield 0.43 g (75%), orange powder, m.p. 97–98 °C. IR: ν 3629, 3373, 3272, 3066 (OH, NH); 1649 (C=O); 1596, 1497, 1480 (C=N, C=C); 1228–1129 (C-F) cm⁻¹. 1H NMR (400 MHz, CDCl₃) δ 2.26 (s, 3H, Me); 7.27 (br. s, 1H, NH); 7.32–7.33, 7.44–7.48, 7.75–7.77 (all m, 5H, Ph); 11.45 (s, 1H, OH). 19F NMR (400 MHz, CDCl₃) δ 100.45 (d, J 0.9 Hz, CF₃). Anal. calcd. for C₁₂H₁₀F₃N₂O₂: C, 50.53; H, 3.53; N, 14.73. Found: C, 50.44; H, 3.40; N, 14.58.

3.1.2. Synthesis of 4-Aminopyrazoles 4a–j (General Procedure)

Method A. Tin (II) chloride dihydrate (8 mmol, 0.18 g) was dissolved in a minimum quantity of concentrated hydrochloric acid, and 4-hydroxyiminopyrazoles 1a–f,i (2 mmol) were added. The mixture was stirred for 2–3 h at r.t. or until a change in color of the initial compounds 1 was observed. The resulting solid was filtered off and washed with n-hexane to yield compounds 4a–f,i.

Method B. A solution of 4-hydroxyiminopyrazoles 1c–e,j (1 mmol) in EtOH (10 mL) and 50 μL of HCl was hydrogenated in the presence of 5 wt.% Pd/C catalyst (10% mmol) in a steel autoclave under a hydrogen pressure of 5–7 bar and r.t. for 4–5 h. The solid impurities were filtered off, and the solvent was removed in vacuo. The resulting compound 4c–e,j was washed with CHCl₃.

4-Amino-3-(trifluoromethyl)-1-phenyl-1H-pyrazol-5-ol hydrate hydrochloride (4a). Yield 0.42 g (78%, method A), orange powder, m.p. 126–127 °C. IR: ν 3574, 3504, 3080, 2935 (OH, NH₂); 1620, 1561, 1539, 1515 (C=N, C=C); 1153–1087 (CF) cm⁻¹. 1H NMR (400 MHz, DMSO-d₆): δ 7.42–7.46, 7.52–7.56, 7.69–7.71 (all m, 5H, Ph); NH₂ and OH are overlapped by Ph protons.

1H NMR (The 1H NMR spectrum was registered along with 13C NMR after standing overnight [M1]) (500 MHz, DMSO-d₆): a mixture of AH:AS (77:23): δ 7.21–7.24 (m, 1H, Ph AS); 7.23 (t, δ 51.0 Hz, NH₃⁺ AS); 7.43–7.47 (m, 1H+2H, Ph AH+AS); 7.53–7.57, 7.69–7.70 (both m, 4H, Ph AH); 7.93–7.94 (m, 2H, Ph AS); 9.42 (br.s, 3H, OH+NH₂ AH). 13C NMR [M1] (500 MHz, DMSO-d₆): a mixture of AH:AS: δ 118.53, 120.57 (q, J 270.4 Hz, CF₃); 122.78 (br. m); 124.27; 124.73; 128.13 (br. m); 128.79; 129.25; 137.14 (q, J 35.1 Hz, C–CF₃); 138.79; 147.06 (br. m); 152.52. 19F NMR (400 MHz, DMSO-d₆): δ 102.35 (s, CF₃). Anal. calcd. for C₁₀H₇ClF₃N₃O: C, 42.95; H, 3.24; N, 15.03; Cl, 12.68. Found: C, 42.96; H, 3.25; N, 15.02; Cl, 12.72.

4-Amino-3-(pentafluoromethyl)-1-phenyl-1H-pyrazol-5-ol hydrate hydrochloride (4b). Yield 0.52 g (75%, method A), light yellow powder, m.p. 176–178 °C. IR: ν 3057, 2884 (OH, NH₂); 1616, 1529, 1506, 1486 (C=N, C=C); 1138–1106 (CF) cm⁻¹. 1H NMR (500 MHz, DMSO-d₆): δ 7.45–7.46, 7.54–7.57, 7.69–7.71 (all m, 5H, Ph); NH₂ and OH are not observed due to deuteron exchange. 1H NMR [M1] (500 MHz, DMSO-d₆): a mixture of AH:AS (40:60): δ 5.60 (br.s, 3H, OH+NH₂ AH); 7.22–7.24 (m, 1H, Ph AS); 7.24 (t, δ 51.0 Hz, NH₃⁺ AS); 7.46–7.49 (m, 2H, Ph AS); 7.49–7.43 (m, 1H, Ph AH); 7.55–7.58, 7.68–7.69 (both m, 4H, Ph AH). 13C NMR [M1] (500 MHz, DMSO-d₆): a mixture of AH:AS: δ 118.33–113.00; 116.80–119.82 (both m, CₓFₓ); 118.47; 121.79; 122.50 (br. m); 124.80; 125.02; 127.02; 127.97 (br. m); 128.81; 129.02; 129.25; 131.59–132.18 (m); 135.93 (t, δ 26.1 Hz, C–CF₃); 137.10 (br. m); 138.11; 138.73; 147.09 (br. m); 152.34. 19F NMR (500 MHz, DMSO-d₆): δ 51.96 (m, 2F, CF₂); 80.07 (t, δ 2.6 Hz, 3F, CF₃). Anal. calcd. for C₁₁H₁₁ClF₃N₃O₂: C, 38.00; H, 3.19; N, 12.09; Cl, 37.79; H, 2.98; N, 11.98; Cl, 10.41.

5-Hydroxy-1-phenyl-3-(nonafluorobutyl)-1H-pyrazol-4-ammonium hydrochloride (4c). Yield 0.56 g (65%, method A), 0.39 g (45%, method B), orange powder, m.p. 174–175 °C. IR: ν 3235, 3143, 3049, 2823 (OH, NH₂); 1586, 1515, 1493, 1460 (C=N, C=C); 1133–1228 (CF) cm⁻¹. 1H NMR (500MHz, DMSO-d₆): δ 7.15 (t, 3H, NH₃⁺, J 51.0 Hz); 7.21–7.25, 7.45–7.48, 7.93–7.94 (all m, 5H, Ph); OH is not observed due to deuteron exchange. 19F NMR (500MHz, DMSO-d₆) δ 36.79–36.84, 40.34–40.40, 51.36–51.40 (all m, 6F; 3 CF₂); 81.95 (t, δ 8.5 Hz, 3F, CF₃). Anal. calcd. for C₁₅H₁₀F₃ClF₃N₃O: C, 36.34; H, 2.11; N, 9.78; Cl, 8.25. Found: C, 36.13; H, 1.99; N, 9.53; Cl, 8.46.

4-Amino-3-methyl-1-phenyl-1H-pyrazol-5-ol hydrochloride hydrate (4d). Yield 0.37 g (77%, method B), orange powder, m.p. 171 °C dec. IR: ν 3441, 3381, 3068, 2839 (OH, NH₂); 1638,
1592, 1540, 1487 (C=N, C=C). 1H NMR (500 MHz, DMSO-d$_6$): δ 2.26 (s, 3H, Me); 7.27–7.30, 7.46–7.50, 7.70–7.71 (all m, 5H, Ph); 10.15 (br.s, 3H, NH$_2$ and OH). 13C NMR (500 MHz, DMSO-d$_6$): δ 11.24; 96.81; 120.20; 125.82; 129.07 (2C); 137.29; 142.87. Anal. calcd. for C$_{10}$H$_{12}$ClN$_3$O$_2$: C, 49.29; H, 5.79; N, 17.24; Cl, 14.55. Found: C, 49.03; H, 5.74; N, 17.26, Cl, 14.68.

4-Amino-3-(trifluoromethyl)-1H-pyrazol-5-ol hydrochloride (4e). Yield 0.31 g (78%, method B). orange powder, m.p. 200–201 °C (lit. [35] m.p. 194–195 °C). IR: ν 3124, 3055, 2872 (OH, NH$_2$); 1586, 1567, 1544, 1505 (C=N, C=C); 1147–1085 (CF) cm$^{-1}$. 1H NMR (400 MHz, DMSO-d$_6$): δ 10.43, 13.39 (both br.s, 4H, OH and NH$_2$). 13C NMR (500 MHz, DMSO-d$_6$): δ 92.76; 120.55 (q, J = 268.9 Hz, CF$_3$); 133.01 (m, C—CF$_3$); 148.84. 19F NMR (400 MHz, DMSO-d$_6$): δ 102.66 (s, CF$_3$). Anal. calcd. for C$_5$H$_5$ClF$_3$N$_3$O: C, 23.60; H, 2.48; N, 20.64; Cl 17.42. Found: C, 23.37; H, 2.44; N, 20.19; Cl 17.56.

4-Amino-3-(1,1,2,2-tetrafluoroethyl)-1H-pyrazol-5-ol hydrate hydrochloride (4f). Yield 0.38 g (75%, method A), white powder, m.p. 144 °C dec. IR: ν 3198, 2963, 2802, 2680 (OH, NH$_2$); 1592, 1571, 1523, 1511 (C=N, C=C); 1152–1089 (CF) cm$^{-1}$. 1H NMR (400 MHz, DMSO-d$_6$): δ 6.84 (t, J = 51.4 Hz, 1H, HCF$_3$); 10.12 (br.s, 3H, NH$_2$ and OH); 13.34 (br.s, 1H, NH). 13F NMR (400 MHz, DMSO-d$_6$): δ 24.90 (m, 2F, CF$_2$); 49.78 (m, 2F, CF$_3$). Anal. calcd. for C$_5$H$_5$ClF$_4$N$_3$O$_2$: C, 23.68; H, 3.18; N, 16.57; Cl 13.98. Found: C, 23.55; H, 2.98; N, 16.31; Cl, 14.05.

4-Amino-3-(pentafluoroethyl)-1H-pyrazol-5-ol hydrate hydrochloride (4g). Yield 0.36 g (66%, method A), white powder, m.p. 160 °C dec. IR: ν 3133, 2956, 2798, 2697 (OH, NH$_2$); 1589, 1570, 1517, 1502 (C=N, C=C); 1147–1103 (CF) cm$^{-1}$. 1H NMR (500 MHz, DMSO-d$_6$): δ 10.56 (brs, 3H, NH$_2$ and OH); 13.56 (br.s, 1H, NH). 19F NMR (500 MHz, DMSO-d$_6$): δ 52.30 (m, 2F, CF$_2$); 79.90 (m, 3F, CF$_3$). Anal. calcd. for C$_5$H$_5$ClF$_5$N$_3$O$_2$: C, 22.11; H, 2.60; N, 15.47; Cl, 13.05. Found: C, 21.78; H, 2.25; N, 15.11; Cl, 13.42.

4-Amino-3-(heptafluoropropyl)-1H-pyrazol-5-ol hydrate hydrochloride (4h). Yield 0.46 g (72%, method A), white powder, m.p. 146 °C subl. IR: ν 3103, 3007, 2942, 2806 (OH, NH$_2$); 1618, 1590, 1571 (C=N, C=C); 1149–1087 (C-F). 1H NMR (400 MHz, DMSO-d$_6$): δ 9.72 (br.s, 3H, NH$_2$ and OH); 13.54 (br.s, 1H, NH). 19F NMR (400 MHz, DMSO-d$_6$): δ 36.89 (m, 2F, β-CF$_2$); 53.80 (m, 2F, α-CF$_2$); 83.06 (t, 3F, CF$_3$ J = 9.0 Hz). Anal. calcd. for C$_5$H$_5$ClF$_7$N$_3$O$_2$: C, 22.41; H, 2.19; N, 13.07; Cl, 11.02. Found: C, 22.22; H, 2.07; N, 12.96; Cl, 11.25.

4-Amino-3-(nonafluorobutyl)-1H-pyrazol-5-ol hydrate hydrochloride (4i). Yield 0.50 g (68%, method A), white powder, m.p. 188–190 °C subl. IR: ν 3512, 3104, 2873, 2615 (OH, NH$_2$); 1589, 1500 (C=N, C=C); 1230–1101 (CF) cm$^{-1}$. 1H NMR (DMSO-d$_6$): δ 10.11 (br.s, 1H, NH). 19F NMR (DMSO-d$_6$): δ 37.48 (m, 2F, γ-CF$_2$); 40.71 (d, d, J = 17.1, 8.2 Hz, 2F, β-CF$_2$); 54.61 (m, 2F, α-CF$_2$); 82.11 (t, J = 9.4 Hz, 3F, CF$_3$). Anal. calcd. for C$_5$H$_5$ClF$_9$N$_3$O$_2$: C, 22.63; H, 1.90; N, 11.31; Cl, 9.54. Found: C, 22.68; H, 1.85; N, 11.11; Cl, 9.39.

4-Amino-methyl-1H-pyrazol-5-ol hydrochloride (4j). Yield 0.20 g (67%, method B), orange powder, m.p. 200 °C dec (lit. [84] m.p. 200–203 °C). IR: 3222, 2877, 2694, 2604 (OH, NH$_2$); 1674, 1639, 1590, 1579, 1556, 1532 (C=N, C=C). 1H NMR (500 MHz, DMSO-d$_6$): δ 2.18 (s, 3H, Me); 9.84, 11.06 (both brs, 3H, NH$_2$ and OH). 13C NMR (500 MHz, DMSO-d$_6$): δ 9.55; 95.47; 134.06; 154.34. Anal. calcd. for C$_5$H$_5$ClN$_3$O: C, 32.12; H, 5.39; N, 28.09; Cl, 23.70. Found: C, 31.94; H, 5.38; N, 28.29; Cl, 23.90.

Copies of NMR spectra of compounds are available in Supplementary Material.

3.2. Biochemical Methods

3.2.1. ABTS Radical Cation Scavenging Activity Assay

Radical scavenging activity of the compounds was assessed using the ABTS radical cation (ABTS$^•$) decolorization assay [56] with some modifications [57]. ABTS (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), and potassium persulfate (diptassium peroxodisulfate), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), and HPLC-grade ethanol and DMSO were obtained from Sigma-Aldrich (Saint Louis, MO, USA). Aque-
ous solutions were prepared using deionized water. All test compounds were dissolved in DMSO.

The solution of cation radical ABTS** was produced by incubation of an aqueous solution of 7 mM ABTS and 2.45 mM potassium persulfate solution in equal quantities for 12–16 h at room temperature in the dark. Radical scavenging capacity of the compounds was analyzed by mixing 10 µL of compound with 240 µL of ABTS** working solution in ethanol (100 µM final concentration). Data were given for 1 h of incubation of compounds with ABTS**. The reduction in absorbance was measured spectrophotometrically at 734 nm using an xMark microplate UV/VIS microplate spectrophotometer (Bio-Rad, Hercules, CA, USA).

Ethanol blanks were run in each assay. Values were obtained from three replicates of each sample and three independent experiments.

Standard antioxidant Trolox was used as a reference compound. Antioxidant capacity as Trolox equivalent (TEAC) values was determined as the ratio between the slopes obtained from the linear correlation of the ABTS radical absorbance with the concentrations of tested compounds and Trolox. Quercetin was used as a positive control. For the test compounds that reduced ABTS** absorbance by more than 60% at 100 µM, we also determined the IC$_{50}$ values (the compound concentration required for a 50% reduction of the ABTS radical). The compounds were tested in the concentration range of 0.5–400 µM.

3.2.2. The FRAP (Ferric Reducing Antioxidant Power) Assay

The FRAP (ferric reducing antioxidant power) assay measures the ability of antioxidants to reduce the ferric 2,4,6-tripyridyl-s-triazine complex [Fe (TPTZ)$_2$]$^{3+}$ to the intensely blue ferrous complex [Fe (TPTZ)$_2$]$^{2+}$ ($\lambda = 593$ nm) in acidic medium [58,59]. 2,4,6-Tris(pyridin-2-yl)-1,3,5-triazine (TPTZ), FeCl$_3$·6H$_2$O, Trolox, quercetin and DMSO were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). The FRAP reagent contained 2.5 mL of 10 mM TPTZ solution in 40 mM HCl, 2.5 mL of 20 mM FeCl$_3$·6H$_2$O water solution, and 25 mL of 0.3 M acetate buffer (pH 3.6). Aliquots of 10 µL of the test compound solution in DMSO (0.5 mM) were mixed with 240 µL of FRAP reagent, and the absorbance of the mixture was measured spectrophotometrically (FLUOStar OPTIMA microplate spectrophotometer (BMG Labtech, Ortenberg, Germany)) at 600 nm after 1 h incubation at 37 °C against a blank. Trolox was used as a reference compound. Quercetin was used as a positive control. A calibration curve with linear formula $y = 0.03x$ ($R^2 = 0.998, p < 0.0001$) was prepared for Trolox solutions in DMSO at a range of 2.5–100 µM, and the results are expressed as Trolox equivalents (TE), the values calculated as the ratio of the concentrations of Trolox and the test compound, resulting in the same effect.

3.2.3. ORAC Assay

AAPH (2,2′-azobis(2-aminopropane) dihydrochloride), Trolox, and fluorescein sodium salt were purchased from Sigma-Aldrich, USA. PBS (phosphate-buffered saline) was from VWR Life Science. DMSO (dimethylsulfoxide) was obtained from Vekton, Russia.

The oxygen radical absorbance capacity (ORAC) was determined by the ability of compounds to protect fluorescein (FL) from peroxyl radicals that are generated by AAPH according to the known method [60,85] with minor variations. The reaction was carried out at 37 °C in black 96-well plates (SPL life sciences), and the final volume of the reaction mixture was 200 µL per well. The fluorescence was recorded by Tecan M1000 PRO microplate reader (Switzerland) at 485 nm excitation and 520 nm emission.

The 0.7 mM fluorescein sodium salt stock solution in PBS pH 7.4 (137 mM sodium chloride, 2.7 mM potassium chloride, and 10 mM phosphate buffer) was prepared and stored at 4–5 °C no longer than one month. An aliquot of the fluorescein stock solution was further diluted with PBS immediately before each assay to yield a 70 nM working solution. The 133 mM AAPH solution in PBS was prepared immediately before use. The test compounds and Trolox standard were dissolved in DMSO to 400 µM. The final
concentration was 10 µM for all of the samples. For IC\textsubscript{50} determination, a set of compound concentrations in the range of 0.5–50 µM was used.

The blank was composed of 15 µL PBS pH 7.4, 5 µL DMSO (final concentration: 2.5% (v/v)), 120 µL FL, and 60 µL AAPH, and it was included in each assay. PBS pH 7.4 (15 µL), antioxidant (the test compound or Trolox, 5 µL), and FL (120 µL, final concentration: 42 nM) solutions were placed in a black 96-well microplate and were pre-incubated for 15 min at 37 °C. AAPH solution (60 µL, final concentration 40 mM) was then added rapidly using a multichannel pipette. The fluorescence was recorded every minute for 2 h. A Trolox standard curve was also obtained in each assay. All reactions were carried out in quadruplicate, and two to four independent assays were performed for each sample.

Antioxidant curves (fluorescence vs. time) were first normalized to the curve of the blank (without antioxidant) corresponding to the same assay, and the net AUC corresponding to a sample was calculated by subtracting the AUC corresponding to the blank. The ORAC value was obtained by dividing the latter curve by the Trolox curve obtained in the same assay at 10 µM concentrations of compounds and Trolox. Final ORAC values, Trolox equivalents (TE), were expressed as µmol test compounds per µmol Trolox, where the value of TE for Trolox was taken as 1. Data were expressed as means ± SEM.

3.2.4. Inhibition In Vitro of Human Erythrocyte AChE, Equine Serum BChE, and Porcine Liver CES

Human erythrocyte acetylcholinesterase (AChE, EC 3.1.1.7), equine serum butyrylcholinesterase (BChE, EC 3.1.1.8), porcine liver carboxylesterase (CES, EC 3.1.1.1), acetylthiocholine iodide (ATCh), butyrylthiocholine iodide (BTrCh), 4-nitrophenol acetate (4-NPA), and 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) were purchased from Sigma-Aldrich (Saint Louis, MO, USA).

All the kinetic experiments were performed under standard conditions, according to the protocol of IPAC RAS for a reversible inhibitors study. Ellman’s colorimetric assay was used to measure AChE and BChE activity in 0.1 M K/Na phosphate buffer at pH 7.5, 25 °C [86]. Final concentrations of reactants were 0.33 mM DTNB, 0.02 unit/mL of AChE or BChE, and 1 mM of substrate (ATCh or BTrCh, respectively). Reagent blanks consisted of reaction mixtures without enzyme to assess non-enzymatic hydrolysis of substrates. Porcine liver CES activity was assessed colorimetrically in 0.1 M K/Na phosphate buffer with pH 8.0 at 25 °C, by measuring the absorbance of 4-nitrophenol at 405 nm [87]. Final enzyme and substrate (4-NPA) concentrations were 0.02 unit/mL and 1 mM, respectively. Reagent blanks included all constituents except enzyme.

Test compounds were dissolved in DMSO. Reaction mixtures contained a final DMSO concentration of 2% (v/v). Enzyme inhibition was first assessed at a single concentration of 20 µM for each compound after a 10 min incubation at 25 °C in three separate experiments. Compounds inhibiting the enzyme by more than 30% were then selected for determination of the IC\textsubscript{50} (inhibitor concentration resulting in 50% inhibition of control enzyme activity). Compounds (eight concentrations ranging between 10\textsuperscript{−11} and 10\textsuperscript{−4} M) were selected to achieve 20 to 80% inhibition and were incubated with each enzyme for 10 min at 25 °C. Substrate was then added, and residual enzyme activity relative to an inhibitor-free control was measured using a FLUOStar Optima microplate reader (LabTech, Ortenberg, Germany).

3.2.5. Cytotoxicity Studies

Cytotoxicity of compounds was evaluated using a culture of human dermal fibroblasts, which were isolated in the Laboratory of Cell Cultures of the Institute of Medical Cell Technologies, Ekaterinburg, Russia. Cells were seeded in 96-well plates in the inoculum dose of 2 × 10\textsuperscript{5} cells/mL and cultured for 24 h in Dulbecco’s Modified Eagle Medium (DMEM), with 1% (w/v) glutamine in the presence of 10% (v/v) fetal bovine serum and gentamicin (50 mg/L) at 37 °C, with a humidified atmosphere of 5% (v/v) CO\textsubscript{2}. Then, compounds,
which were solved in DMSO, were added to the wells at the final concentrations of 0.1, 1, 10, and 100 µM. The cells were incubated with compounds for 72 h, after which cell viability was assessed using the standard MTT assay [81]. The assay was carried out in four replicates with negative (culture medium) and positive (solution of the cytotoxic drug camptothecin at a concentration of 3 mM) controls, and the solvent control (DMSO). The results of the MTT assay were evaluated on a Tecan Infinite M200 PRO (Tecan Austria GmbH, Grödig, Austria) plate spectrophotometer by comparing the optical density of a formazan solution at 570 nm in the assay and control wells. The MTT staining of control cells was taken as 100%.

3.3. Quantum-Chemical Calculations

Adiabatic and vertical indices (electron affinity $EA$, ionization potential $IP$, and derived values) for 4-hydroxyiminopyrazolones 1a–j were evaluated in the gas phase approximation (T 298 K and a pressure of 1 atm). The electronic parameters of the salts of 4-aminopyrazololes 4a–j were calculated in the presence of the solvent (aqueous ethanol), because the effect of the solvent on cations is more pronounced than on neutral molecules. To obtain vertical values of electronic parameters, single-point calculations of anion and cation radicals were performed in the equilibrium geometry of the most stable conformer of a neutral species. In the case of salts 4a–j, the cation structures were fully optimized, whereas single-point calculations were made for neutral or doubly charged species to estimate the $EA$ or $IP$ quantities, respectively. These indices for the studied compounds were calculated according to [88] using the following equations:

$$\eta = \frac{1}{2} (IP - EA) \quad (1)$$

where $\eta$ = the chemical hardness.

The ionization potential was estimated as the difference between the energies for the closed-shell N-electron and the open-shell $(N - 1)$-electron species [89]:

$$IP = E(A^{++}) - E(A) \quad (2)$$

The electron affinity of a molecule was determined in a similar manner using the open-shell $(N + 1)$-electron species:

$$EA = E(A) - E(A^{+-}) \quad (3)$$

where $E$ is the total energy $E_{tot}$ in the calculations of vertical values.

3.4. Statistical Analyses

Experimental data were expressed as means ± SEM ($n = 3$ independent experiments) using GraphPad Prism version 6.05 for Windows, GraphPad Software (San Diego, CA, USA). Linear regressions with coefficients of determination ($R^2$) were determined using Origin 6.1 for Windows (OriginLab, Northampton, MA, USA). Data were tested for normality (Gaussian distribution) using the D’Agostino–Pearson, Anderson–Darling, Shapiro–Wilks, and Kolmogorov–Smirnov tests as implemented in GraphPad Prism 9.4.1 for Windows GraphPad software (San Diego, CA, USA). The data from the ORAC antioxidant test passed all four normality tests, while the data from the FRAP antioxidant tests failed all four normality tests, and the data from the ABTS antioxidant test failed the Anderson–Darling normality test. Therefore, determinations of correlation coefficients between the three pairs of antioxidant data were carried out using the non-parametric Spearman $\rho$ rather than the parametric Pearson $r$, again using Prism 9.4.1 for Windows. Statistical significance was set at $p < 0.05$. 
4. Conclusions

We have proposed effective approaches for the reduction of 4-hydroxyiminopyrazol-5-ones, which allowed us to obtain a series of new 4-aminopyrazol-5-ols as analogs of Edaravone. It was found that 4-aminopyrazol-5-ols can exist only as salts, while in free form they either decompose or transform into imino-bis-pyrazolols (rubazonic acids). The structure of the resulting aminopyrazolol salts was confirmed by IR and NMR spectroscopy along with elemental analysis.

The antioxidant action of 4-aminopyrazol-5-ol hydrochlorides and their precursors—4-hydroxyiminopyrazol-5-ones—was investigated in three test systems (ABTS, FRAP, and ORAC) in comparison with EDA, CF$_3$-EDA, and Trolox. These studies revealed a pronounced antioxidant activity of aminopyrazolols in all tests. The lead compound was 4-amino-3-methyl-1-phenylpyrazol-5-ol hydrochloride (4d). Its activity in ABTS and FRAP tests was comparable to that of EDA, CF$_3$-EDA, and Trolox, whereas in the ORAC test, it showed higher efficacy than the reference compounds. The similar character of the change in antioxidant activity values with the variation in the structure of the compounds in the ABTS and FRAP experiments allowed us to conclude that the transformations of the test compounds in these tests occurred by the SET mechanism, while in the ORAC test, the transformations took place via the HAT mechanism.

Using quantum-chemical calculations, in general, the salts of 4-aminopyrazolols 4 were found to be characterized by lower values of the calculated electronic characteristics [gap, ionization potential (IP), and chemical hardness ($\eta$)] [89] compared to 4-hydroxyiminopyrazol-5-ones 1, and these results are consistent with their higher antioxidant activities determined experimentally in the ABTS and FRAP tests. Calculations showed that the most energetically favorable pathway was the formation of radicals through HO bond breaking in both 4-hydroxyiminopyrazolones 1 and 4-aminopyrazolols 4. The transformations of 4-aminopyrazolols 4 bearing more groups that are prone to the formation of radicals were not as unambiguous and more dependent on structural fragments. Thus, the antioxidant activity of PhN-4-aminopyrazolols 4a–d in the ABTS test was determined by gap functions, whereas, for HN-unsubstituted analogs, correlation with IP functions was found only for perfluoroalkyl derivatives 4e,h–i. The antioxidant activity in the ORAC test directly depended on the OH bond dissociation energy for active 4-hydroxyiminopyrazolones 1e–j and for all group of 4-aminopyrazolols 4a–j.

The esterase profile results demonstrated the absence of anticholinesterase activity for all the compounds and a moderate inhibition of CES by some 4-aminopyrazolols 4, especially for compounds 4c and 4i with a nonafluorobutyl substituents. The CES result should be taken into consideration in the potential therapeutic use of these compounds in high doses. In addition, the lead compound 4d and its NH-analog 4j did not show cytoxicity against normal human fibroblasts, in contrast to EDA.

Thus, the investigation showed that 4-aminopyrazol-5-ols are promising antioxidant agents with activity at the Edaravone level and above. In particular, the lead compound 4d displayed good antioxidant properties, indicating its promise for potential future use as a novel therapeutic drug candidate in the treatment of diseases associated with oxidative stress. Moreover, the new synthesized aminopyrazolols are of interest for further modification as antioxidant pharmacophores, e.g., for conjugation to an anticholinesterase fragment and for the creation of multifunctional drugs for the treatment of neurodegenerative diseases.

Supplementary Materials: The following information is available online at https://www.mdpi.com/article/10.3390/molecules27227722/s1: Figure S1: $^1$H NMR spectrum of bis[5-hydroxy-1-phenyl-3-(trifluoromethyl)-1H-pyrazol-4-yl]imine (2a); Figure S2: $^{13}$C NMR spectrum of bis[5-hydroxy-1-phenyl-3-(trifluoromethyl)-1H-pyrazol-4-yl]imine (2a); Figure S3: $^{19}$F NMR spectrum of bis[5-hydroxy-1-phenyl-3-(trifluoromethyl)-1H-pyrazol-4-yl]imine (2a); Figure S4: $^1$H NMR spectrum of bis[5-hydroxy-1-phenyl-3-(trifluoromethyl)-1H-pyrazol-4-yl]imine (2a); Figure S5: $^{19}$F NMR spectrum of bis[5-hydroxy-1-phenyl-3-(trifluoromethyl)-1H-pyrazol-4-yl]imine (2a); Figure S6: $^1$H NMR spectrum of N-(5-hydroxy-3-(trifluoromethyl)-1-phenyl-1H-pyrazol-4-yl)acetamide (3a); Figure S7: $^{19}$F NMR spectrum of N-(5-hydroxy-3-(trifluoromethyl)-1-phenyl-1H-pyrazol-4-yl)acetamide (3a).
Neha, K.; Haider, M.R.; Pathak, A.; Yar, M.S. Medicinal prospects of antioxidants: A review. *Eur. J. Med. Chem.* 2019, 178, 687–704. [CrossRef] [PubMed]

Liu, Z.-Q. Bridging free radical chemistry with drug discovery: A promising way for finding novel drugs efficiently. *Eur. J. Med. Chem.* 2018, 156, 394–429. [CrossRef] [PubMed]

Silva, V.L.M.; Elguero, J.; Silva, A.M.S. Current progress on antioxidants incorporating the pyrazole core. *Eur. J. Med. Chem.* 2018, 156, 394–429. [CrossRef] [PubMed]
4. Zhao, Z.; Dai, X.; Li, C.; Wang, X.; Tian, J.; Feng, Y.; Xie, J.; Ma, C.; Nie, Z.; Fan, P.; et al. Pyrazolone structural motif in medicinal chemistry: Retrospect and prospect. *Eur. J. Med. Chem.* 2020, 186, 111893. [CrossRef]

5. Queiroz, A.N.; Martins, C.C.; Santos, K.L.B.; Carvalho, E.S.; Owitti, A.O.; Oliveira, K.R.M.; da Silva, A.B.F.; Borges, R.S. Experimental and theoretical study on structure-tautomerism among edaravone, isoxazolone, and their heterocycles derivatives as antioxidants. *Saudi Pharm. J.* 2020, 28, 819–827. [CrossRef] [PubMed]

6. Abe, K.; Yuki, S.; Kogure, K. Strong attenuation of ischemic and postischemic brain edema in rats by a novel free radical scavenger. *Stroke* 1988, 19, 480–485. [CrossRef]

7. Miyaji, Y.; Yoshimura, S.; Sakai, N.; Yamagami, H.; Egashira, Y.; Shirakawa, M.; Uchida, K.; Kageyama, H.; Tomogane, Y. Effect of Edaravone on Favorable Outcome in Patients with Acute Cerebral Large Vessel Occlusion: Subanalysis of RESCUE-Japan Registry. *Neurot. Med. Chir.* 2015, 55, 241–247. [CrossRef]

8. Sun, Z.; Xu, Q.; Gao, G.; Zhao, M.; Sun, C. Clinical evaluation of edaravone treatment in acute cerebral infarction. *Niger. J. Clin. Pract.* 2019, 22, 1324–1327. [CrossRef]

9. Dash, R.P.; Babu, R.J.; Srinivas, N.R. Two Decades-Long Journey from Riluzole to Edaravone: Revisiting the Clinical Pharmacokinetics of the Only Two Amyotrophic Lateral Sclerosis Therapeutics. *Clin. Pharmacokinet.* 2018, 57, 1385–1398. [CrossRef] [PubMed]

10. Luo, L.; Song, Z.; Li, X.; Huiwang; Zeng, Y.; Qinwang; Meiqi; He, J. Efficacy and safety of edaravone in treatment of amyotrophic lateral sclerosis—a systematic review and meta-analysis. *Neuroly. Sci.* 2018, 40, 235–241. [CrossRef]

11. Shenefr, J.; Heiman-Patterson, T.; Fiorio, E.P.; Wiedau-Pazos, M.; Liu, S.; Zhang, J.; Agnese, W.; Apple, S. Long-term edaravone efficacy in amyotrophic lateral sclerosis: Post-hoc analyses of Study 19 (MCI186-19). *Muscle Nerve* 2019, 61, 218–221. [CrossRef] [PubMed]

12. Bailly, C. Potential use of edaravone to reduce specific side effects of chemo-, radio- and immuno-therapy of cancers. *Int. Immunopharmacol.* 2019, 77, 105967. [CrossRef]

13. Zhang, W.-W.; Bai, F.; Wang, J.; Zheng, R.-H.; Yang, L.-W.; James, E.; Zhao, Z.-Q. Edaravone inhibits pressure overload-induced cardiac fibrosis and dysfunction by reducing expression of angiotensin II AT1 receptor. *Drug Des. Dev. Ther.* 2017, 11, 3019–3033. [CrossRef] [PubMed]

14. Zhao, Z.-Q.; Pang, X.-F.; Zhang, L.-H.; Bai, F.; Wang, N.-P.; McKallip, R.; Garner, R. Attenuation of myocardial fibrosis with curcumin is mediated by modulating expression of angiotensin II AT1 and AT2 receptors and ACE2 in rats. *Drug Des. Dev. Ther.* 2015, 9, 6034–6045. [CrossRef] [PubMed]

15. Kikuchi, K.; Takeshige, N.; Miura, N.; Morimoto, Y.; Ito, T.; Tancharoen, S.; Miyata, K.E.I.; Kikuchi, C.; Iida, N.; Uchikado, H.; et al. Beyond free radical scavenging: Beneficial effects of edaravone (Radicut) in various diseases (Review). *Exp. Ther. Med.* 2012, 3, 3–8. [CrossRef]

16. Raymer, B.; Bhattacharya, S.K. Lead-like Drugs: A Perspective. *J. Med. Chem.* 2018, 61, 10375–10384. [CrossRef] [PubMed]

17. Higashi, Y.; Jitsuiki, D.; Chayama, K.; Yoshizumi, M. Edaravone (3-Methyl-1-Phenyl-2-Pyrazolin-5-one), A Novel Free Radical Scavenger, for Treatment of Cardiovascular Diseases. *Recent Pat. Cardiovasc. Drug Discov.* 2006, 1, 85–93. [CrossRef] [PubMed]

18. Ren, Y.; Wei, B.; Song, X.; An, N.; Zhou, Y.; Jin, X.; Zhang, Y. Edaravone’s free radical scavenging mechanisms of neuroprotection against cerebral ischemia: Review of the literature. *Int. J. Neurosci.* 2014, 125, 555–565. [CrossRef]

19. Kamogawa, E.; Sueishi, Y. A multiple free-radical scavenging (MULTIS) study on the antioxidant capacity of a neuroprotective drug, edaravone as compared with uric acid, glutathione, and trolox. *Bioorg. Med. Chem. Lett.* 2017, 27, 49–54. [CrossRef] [PubMed]

20. Yamamoto, Y.; Kuwahara, T.; Watanabe, K.; Watanabe, K. Antioxidant activity of 3-methyl-1-phenyl-2-pyrazolin-5-one. *J. Heterocycl. Chem.* 2018, 54, 331–340. [CrossRef]

21. Homma, T.; Kobayashi, S.; Sato, H.; Fujii, J. Edaravone, a free radical scavenger, protects against ferroptotic cell death in vitro. *Exp. Cell Res.* 2019, 384, 111592. [CrossRef] [PubMed]

22. Bailly, C.; Hecquet, P.-E.; Kouach, M.; Thuru, X.; Goossens, J.-F. Chemical reactivity and uses of 1-phenyl-3-methyl-5-pyrazolone (PMP), also known as edaravone. *Bioorg. Med. Chem.* 2020, 28, 115463. [CrossRef] [PubMed]

23. Pérez-González, A.; Galano, A. OH Radical Scavenging Activity of Edaravone: Mechanism and Kinetics. *J. Phys. Chem. B* 2010, 114, 1306–1314. [CrossRef] [PubMed]

24. Yamamoto, Y. Plasma marker of tissue oxidative damage and edaravone as a scavenger drug against peroxyl radicals and peroxynitrite. *J. Clin. Biochem. Nutr.* 2017, 60, 49–54. [CrossRef] [PubMed]

25. Nakagawa, H.; Ohyama, R.; Kimata, A.; Suzuki, T.; Miyata, N. Hydroxyl radical scavenging by edaravone derivatives: Efficient scavenging by 3-methyl-1-(pyridin-2-yl)-5-pyrazolone with an intramolecular base. *Bioorg. Med. Chem. Lett.* 2006, 16, 5939–5942. [CrossRef] [PubMed]

26. Chegaev, K.; Cena, C.; Giorgis, M.; Rolando, B.; Tosco, P.; Bertinaria, M.; Fruttero, R.; Carrupt, P.-A.; Gasco, A. Edaravone Derivatives Containing NO-Donor Functions. *J. Med. Chem.* 2008, 52, 574–578. [CrossRef]

27. Rolando, B.; Filieri, A.; Chegaev, K.; Lazzarato, L.; Giorgis, M.; De Nardi, C.; Fruttero, R.; Martel, S.; Carrupt, P.-A.; Gasco, A. Synthesis physicochemical profile and PAMPA study of new NO donor edaravone co-drugs. *Bioorg. Med. Chem.* 2012, 20, 841–850. [CrossRef]

28. Gaffer, H.E.; Abdel-Fattah, S.; Eltman, H.A.; Abdel-Latif, E. Synthesis and Antioxidant Activity of Some New Thiazolyl-Pyrazolone Derivatives. *J. Heterocycl. Chem.* 2017, 54, 331–340. [CrossRef]
53. Burgart, Y.V.; Agafonova, N.A.; Shchegolkov, E.V.; Krasnykh, O.P.; Kushch, S.O.; Evstigneeva, N.P.; Gerasimova, N.A.; Maslov, V.V.; Triandafilova, G.A.; Solodnikov, S.Y.; et al. Multiple biological active 4-aminoypyrazoles containing trifluoromethyl and their 4-nitroso-precurors: Synthesis and evaluation. *J. Med. Chem. 2020*, *208*, 112768. [CrossRef] [PubMed]

54. Hänself, W. 4-(5-Hydroxy-4-pyrazolylamin)-2-pyrazolin-5-one and its Metalchelate, I Synthese von 4-(5-Hydroxy-4-pyrazolylamin)-2-pyrazolin-5-onen (Rubazonsäuren) und strukturanalog Verbindungen. *Justus Liebigs Ann. Der Chem.* 1976, *1976*, 1380–1394. [CrossRef]

55. Ueda, T.; Oda, N.; Ito, I. Studies on synthetic methods for 5-amino-4(3H)-pyrimidones. I. A novel ring expansion reaction of 4-aminoantipyrines to 5-amino-4(3H)-pyrimidones. *Chem. Pharm. Bull.* 1980, *28*, 2144–2147. [CrossRef]

56. Re, R.; Pellegrini, N.; Prosegger, A.; Fannala, A.; Yang, M.; Rice-Evans, C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radic. Biol. Med.* 1999, *26*, 1231–1237. [CrossRef]

57. Makhaeva, G.F.; Elkina, N.A.; Shchegolkov, E.V.; Boltneva, N.P.; Lushchekina, S.V.; Serebryakova, O.G.; Rudakova, E.V.; Kovaleva, N.V.; Radchenko, E.V.; Palyulin, V.A.; et al. Synthesis, molecular docking, and biological evaluation of 3-oxo-2-tolylhydrazinylidine-4,4,4-trifluorobutanoates bearing higher and natural alcohol moieties as new selective carboxylesterase inhibitors. *Bioorg. Chem.* 2019, *91*, 103097. [CrossRef] [PubMed]

58. Benzie, I.F.F.; Strain, J.J. [2] Ferric reducing/antioxidant power assay: Direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. *Methods Enzymol.* 1999, *299*, 15–27. [CrossRef]

59. Makhaeva, G.F.; Kovaleva, N.V.; Rudakova, N.P.; Rudakova, E.V.; Boltneva, N.P.; Lushchekina, S.V.; Faingold, I.I.; Poletaeva, D.A.; Soldatova, Y.V.; Kotelnikova, R.A.; Serkov, I.V.; et al. New Multifunctional Agents Based on Conjugates of 4-Amino-2,3-polyethylenequinoline and Butylated Hybrid Formulations for Alzheimer’s Disease Treatment. *Molecules* 2020, *25*, 5891. [CrossRef]

60. Ou, B.; Hampsch-Woodill, M.; Prior, R.L. Development and Validation of an Improved Oxygen Radical Absorbance Capacity (ORAC) Assay Using Fluorescein as the Fluorescent Probe. *J. Agric. Food. Chem.* 2001, *49*, 4619–4626. [CrossRef]

61. Gotsbacher, M.P.; Telfer, T.J.; Witting, P.K.; Double, K.L.; Finkelstein, D.I.; Codd, R. Analogues of desferrioxamine B designed to attenuate iron-mediated neurodegeneration: Synthesis, characterisation and activity in the MPTP-mouse model of Parkinson’s disease. *Metallomics* 2017, *9*, 852–864. [CrossRef]

62. Kraus, R.L.; Pasieczny, R.; Lariosa-Willingham, K.; Turner, M.S.; Jiang, A.; Trauger, J.W. Antioxidant properties of minocycline: Neuroprotection in an oxidative stress assay and direct radical-scavenging activity. *J. Neurochem.* 2005, *94*, 819–827. [CrossRef] [PubMed]

63. Makhaeva, G.F.; Rudakova, E.V.; Serebryakova, O.G.; Aksenenko, A.Y.; Lushchekina, S.V.; Bachurin, S.O.; Richardson, R.J. Esterase profiles of organophosphorus compounds in vitro predict their behavior in vivo. *Chem. Biol. Interact.* 2016, *259*, 332–342. [CrossRef] [PubMed]

64. Makhaeva, G.F.; Radchenko, E.V.; Palyulin, V.A.; Rudakova, E.V.; Aksenenko, A.Y.; Sokolov, V.B.; Zefirov, N.S.; Richardson, R.J. Organophosphorus compound esterase profiles as predictors of therapeutic and toxic effects. *Chem. Biol. Interact.* 2013, *203*, 231–237. [CrossRef] [PubMed]

65. Makhaeva, G.F.; Lushchekina, S.V.; Boltneva, N.P.; Serebryakova, O.G.; Kovaleva, N.V.; Rudakova, E.V.; Elkina, N.A.; Shchegolkov, E.V.; Burgart, Y.V.; Stupina, T.S.; et al. Novel potent bifunctional carboxylesterase inhibitors based on a polyfluoroalkyl-2-imino-1,3-dione scaffold. *Eur. J. Med. Chem.* 2021, *218*, 113385. [CrossRef] [PubMed]

66. Makhaeva, G.F.; Boltneva, N.P.; Lushchekina, S.V.; Serebryakova, O.G.; Stupina, T.S.; Terentiev, A.A.; Serkov, I.V.; Proshin, A.N.; Bachurin, S.O.; Richardson, R.J. Synthesis, molecular docking and biological evaluation of N,N-disubstituted 2-aminothiazolines as a new class of butyrylcholinesterase and carboxylesterase inhibitors. *Bioorg. Med. Chem.* 2016, *24*, 1050–1062. [CrossRef]

67. Prior, R.L.; Wu, X.; Schaich, K. Standardized Methods for the Determination of Antioxidant Capacity and Phenolics in Foods and Dietary Supplements. *J. Agric. Food. Chem.* 2005, *53*, 4290–4302. [CrossRef] [PubMed]

68. Jiménez, A.; Selga, A.; Torres, J.L.; Julià, L. Reducing Activity of Polyphenols with Stable Radicals of the TTM Series. Electron Transfer versus H-Abstraction Reactions in Flavan-3-ols. *Org. Lett.* 2004, *6*, 4583–4586. [CrossRef]

69. Lespade, I.; Bercion, S. Theoretical investigation of the effect of sugar substitution on the antioxidant properties of flavonoids. *Free Radic. Res.* 2012, *46*, 346–358. [CrossRef]

70. Mohajeri, A.; Asemani, S.S. Theoretical investigation on antioxidant activity of vitamins and phenolic acids for designing a novel antioxidant. *J. Mol. Struct.* 2009, *930*, 15–20. [CrossRef]

71. Amić, D.; Lučić, B. Reliability of bond dissociation enthalpy calculated by the PM6 method and experimental TEAC values in antiradical QSAR of flavonoids. *Bioorg. Med. Chem.* 2010, *18*, 28–35. [CrossRef]

72. Marković, Z.; Milenković, D.; Dorović, J.; Dimitrić Marković, J.M.; Stepanić, V.; Lučić, B.; Amić, D. PM6 and DFT study of free radical scavenging activity of morin. *Food Chem.* 2012, *134*, 1754–1760. [CrossRef]

73. Zhang, D.; Liu, Y.; Chu, L.; Wei, Y.; Wang, D.; Cai, S.; Zhou, F.; Ji, B. Relationship Between the Structures of Flavonoids and Oxygen Radical Absorbance Capacity Values: A Quantum Chemical Analysis. *J. Phys. Chem. A* 2013, *117*, 1784–1794. [CrossRef] [PubMed]

74. Frisch, M.J.; Trucks, G.W.; Schlegel, H.B.; Scuseria, G.E.; Robb, M.A.; Cheeseman, J.R.; Scalmani, G.; Barone, V.; Petersson, G.A.; Nakatsuji, H.; et al. *Gaussian 09*, Revision C.01; Gaussian Inc.: Wallingford, CT, USA, 2010.

75. Becke, A.D. Density-functional thermochemistry. III. The role of exact exchange. *J. Chem. Phys.* 1993, *98*, 5648–5652. [CrossRef]
76. Davidson, E.R. Comment on “Comment on Dunning’s correlation-consistent basis sets”. Chem. Phys. Lett. 1996, 260, 514–518. [CrossRef]
77. Tomasi, J.; Mennucci, B.; Cammi, R. Quantum Mechanical Continuum Solvation Models. Chem. Rev. 2005, 105, 2999–3094. [CrossRef]
78. Horton, W.; Peerannawar, S.; Török, B.; Török, M. Theoretical and experimental analysis of the antioxidant features of substituted phenol and aniline model compounds. Struct. Chem. 2018, 30, 23–35. [CrossRef]
79. Klein, E.; Lukeš, V.; Cibulková, Z.; Polovková, J. Study of N–H, O–H, and S–H bond dissociation enthalpies and ionization potentials of substituted anilines, phenols, and thiophenols. J. Mol. Struct. 2006, 758, 149–159. [CrossRef]
80. Saqib, M.; Mahmood, A.; Akram, R.; Khalid, B.; Afzal, S.; Kamal, G.M. Density functional theory for exploring the structural characteristics and their effects on the antioxidant properties. J. Pharm. Appl. Chem. 2015, 1, 65–71. [CrossRef]
81. Mosmann, T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J. Immunol. Methods 1983, 65, 55–63. [CrossRef]
82. Dolomanov, O.V.; Bourhis, L.J.; Gildea, R.J.; Howard, J.A.K.; Puschmann, H. OLEX2: A complete structure solution, refinement and analysis program. J. Appl. Crystallogr. 2009, 42, 339–341. [CrossRef]
83. Sheldrick, G.M. A short history ofSHELX. Acta Crystallogr. Sec. A 2007, 64, 112–122. [CrossRef] [PubMed]
84. Ishimaru, T. Study on Chemotherapeutics. III. Yakugaku Zasshi 1957, 77, 800–802. [CrossRef]
85. Jo, S.-H.; Ka, E.-H.; Lee, H.-S.; Apostolidis, E.; Jang, H.-D.; Kwon, Y.-I. Comparison of antioxidant potential and rat intestinal α-glucosidases inhibitory activities of quercetin, rutin, and isoquercetin. Int. J. Appl. Res. Nat. Prod. 2009, 2, 52–60.
86. Ellman, G.L.; Courtney, K.D.; Andres, V.; Featherstone, R.M. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 1961, 7, 88–95. [CrossRef]
87. Sterri, S.H.; Johnsen, B.A.; Fonnum, F. A radiochemical assay method for carboxylesterase, and comparison of enzyme activity towards the substrates methyl [1-14C] butyrate and 4-nitrophenyl butyrate. Biochem. Pharmacol. 1985, 34, 2779–2785. [CrossRef]
88. Pearson, R.G. Hardness of Closed Systems. In Chemical Reactivity Theory: A Density Functional View; Chattaraj, P.K., Ed.; CRC Press: Boca Raton, FL, USA, 2009; pp. 155–162.
89. Cramer, C.J. Essentials of Computational Chemistry: Theories and Models, 2nd ed.; John Wiley & Sons: Chichester, UK, 2004; pp. 330–331.