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A Novel 1,2,3-Triazole Ligand with Antioxidant Activity

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Abstract: The 1,2,3-triazole heterocycle serves as an important pharmacophore in large numbers of drug molecules with disparate pharmacological activities – antiviral, antibacterial, antifungal, anticancer, anticoagulant, CNS-active, to name a few. Its chemical stability, dipole moment, and ability to participate in hydrogen bonding make it an important building block for novel biologically active chemical species. The present work investigates the free-radical scavenging activity of 2-(4-methoxyphenyl)-5-(pyrrolidin-1-yl)-2H-1,2,3-triazole-4-carboxylic acid and its sodium salt. A number of free radical-generating model systems were utilized in order to estimate its potential *in vitro* antioxidant activity. UV-induced water radiolysis and Fenton reaction were used as sources of the physiologically important hydroxyl radical. The observed radical-scavenging activities were additionally investigated with the aid of the stable free radical model systems DPPH and ABTS in order to elucidate the possible mechanisms of radical-scavenging action. Results show that the compounds under investigation behaved as strong scavengers of hydroxyl radicals generated by UV-induced radiolysis. The effect on the Fenton reaction was close to zero or even mildly pro-oxidant; DPPH and ABTS assays showed moderate activity.

Keywords: 1,2,3-triazole; antioxidant; hydroxyl radicals; DPPH; ABTS.

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1. Introduction

Within all living systems, various chemical species containing an unpaired electron are constantly being produced and eliminated via several pathways. Such species, also known as reactive species (RS), appear as a result of electron-transfer reactions associated with a multitude of biochemical processes of life [1]. Their effect can be both helpful and harmful to human health [2]. The impaired balance between RS elimination and excessive RS generation is ubiquitously known as oxidative stress (OS) [3]. OS is associated with and involved in a large number of pathologies [4]. Among the wide variety of RS that are being formed in living organisms, two of the most chemically active, hence harmful, are the hydroxyl radical ($OH^{\bullet}$) and the hydroperoxyl radical ($OOH^{\bullet}$) [5]. They tend to attack various molecular sites, especially those that contain conjugated double bonds [6]. They are associated with the so-called lipid peroxidation – a process of degradation of polyunsaturated lipids in which $OH^{\bullet}$ and $OOH^{\bullet}$ initiate a radical chain reaction that, unless prevented/stopped, produces lipid peroxides and, finally, breaks down lipids to malondialdehyde (MDA) and other MDA-like products [6]. Lipid

peroxidation and the associated chain reactions can be prevented with the help of antioxidants. Antioxidants are molecules that are able to interact with RS and, via hydrogen atom transfer (HAT) or electron transfer reactions, "neutralize" the unpaired electrons of RS, thus making them less harmful to living systems [7]. One type of antioxidant is the so-called radical scavenger [8]. Radical scavenging is a non-enzymatic process of interaction between a free radical and another molecule, as a result of which both are irreversibly transformed into chemically inactive species. Scavengers generally incorporate conjugated p-π and π-π systems that allow significant delocalization of electron density and hence – delocalization of an unpaired electron, thus decreasing their chemical activity [9].

The 1,2,3-triazole heterocycle is an important pharmacophore [10], a component of numerous drugs belonging to disparate pharmacological classes – antibacterial[11], antimalarial [12], and antiproliferative [13], to name a few. It is characterized by a significant dipole moment [14], high chemical stability [15], and the ability to participate in hydrogen bonding. These characteristics imbue the 1,2,3-triazole moiety with the ability to interact with chemical receptors in living organisms [16-18], making it a very attractive "skeleton" for synthesizing diverse, pharmacologically active molecules.

The present work deals with an *in-vitro* investigation of the impact of two triazole derivatives - sodium 2-(4-methoxyphenyl)-5-(pyrrolidin-1-yl)-2H-1,2,3-triazole-4-carboxylate and 2-(4-methoxyphenyl)-5-(pyrrolidin-1-yl)-2H-1,2,3-triazole-4-carboxylic acid (named BNP02 and BNP04, respectively) - on several free radical-generating model systems – UV-induced degradation of 2-deoxyribose, Fenton reaction, 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$) and the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonicfree radical ($ABTS^{\bullet+}$). BNP02 is the conjugate base of BNP04. The structural formulae of both substances are presented in Figure 1.

COONa
N
N
N
N
H_3CO
BNP02

COOH
N
N
N
N
H_3CO
BNP04

Figure 1. Structural formulae of the investigated substances.

Both the carboxylic acid and its sodium salt were synthesized [16] as part of a series of novel fluorescent compounds with potential utility as ligands in lanthanide transition metal complexes with biological activity. The present work discussed results from preliminary investigations into the biological activity of the ligand BNP02.

2. Materials and Methods

All materials were of the highest grade (pro analysis) SIGMA-ALDRICH (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany). Bi-distilled water and 95% ethanol were used as solvents. Stock water solutions of both investigated substances were prepared (BNP02 – 1×10^{-3} M; BNP04 – 1×10^{-4} M due to limited water solubility), applied, and further diluted, if necessary, during experimentation. The following molarities of each substance were tested: 1×10^{-4} M (BNP02), 3×10^{-5} M, 1×10^{-5} M, 3×10^{-6} M, and 1×10^{-6} M (BNP02 and BNP04). For the deoxyribose degradation assay, the following solutions were prepared: 6.0 mM water

solution of deoxyribose; 1% water solution of thiobarbituric acid (TBA); 3% water solution of trichloroacetic acid (TCA); 50 mM K-Na phosphate saline solution (PBS) with pH=7.4. For the Fenton reaction assay, the following water solutions were prepared: 6 mg $FeCl_3$ was added to 10 mL EDTA (4 mg/ 100 mL). To the resulting solution was added 30 μL 30 % H_2O_2. The reagent obtained was prepared on the day of experimentation and was kept in an ice bath. 30 mg/ 10 mL water solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 40 mg/ 10 mL ascorbic acid were also prepared. The antioxidant capacity by way of HAT was investigated using a stock ethanol solution of 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^•$), prepared according to well-established protocols [17,18]. The electron-exchange potential of the substances was investigated using the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic free radical ($ABTS^{•+}$) assay. For that purpose, Erel et al. [19,20] prepared the following solutions: 0.4 mmol acetate buffer, pH-5.8 (R1); 0.03 mmol acetate buffer solution, pH=3.6 with ABTS and H_2O_2 in order to produce the stable $ABTS^{•+}$ (R2).

A Shimadzu UV1601 spectrophotometer was utilized for all UV-VIS measurements. Data from the Fenton reaction, DPPH, and ABTS assays was recorded after 900 s incubation.

For each tested concentration, three parallel measurements were taken, each one representing an individual data point. Averages and standard deviations were calculated. Relative changes within the limits of experimental error were not discussed. The impact of the varying concentrations on the results obtained was statistically verified using one-way ANOVA, followed by Bonferroni post-test.

2.1. Deoxyribose degradation assay.

The investigation utilized a modified protocol by Halliwell et al. [21]. $OH^•$ and $OOH^•$ are generated by UV-induced water radiolysis [22]. The extent of radical-induced degradation of 2-deoxyribose to MDA-like products is estimated by way of the colorimetric reaction of the latter with TBA, resulting in a product with an absorbance maximum at λ=532 nm. Each test sample contained 0.50 mL deoxyribose, the necessary volumes of substance stock solution, and PBS (up to 5.00 mL). The control samples did not contain the tested substance. After 30 minutes of irradiation under a UV lamp (λ= 220-400 nm) from each of the irradiated solutions, 1.00 mL volumes were taken. 0.60 mL TCA and 0.60 mL TBA were added to each volume. Thus prepared, all samples were vortexed and cultivated for 30 minutes in a 100°C water bath. After cultivation, the absorbance of the chromophore was measured at λ=532 nm. The degree of 2–deoxyribose degradation was calculated as a Radical-Scavenging Activity (RSA, %):

$$RSA, \% = \frac{A_{control} - A_{sample}}{A_{control}} * 100 \qquad (1)$$

2.2. Fenton reaction (MTT) assay.

The reduction of the yellow tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to purple formazan was utilized to assess the impact of the ligand and both complexes on Fenton reaction-generated hydroxyl radicals. The sample composition is presented in Table 1.

Table 1. Sample compositions for the Fenton reaction assay.

Reagent	Control	Sample
BNP02/BNP04	no	200 μL
MTT	200 μL	200 μL

Reagent	Control	Sample
$Fe^{2+}/H_2O_2/Na_2$-EDTA	100 μL	100 μL
Ascorbic acid	100 μL	100 μL
Bi-destilled water	up to 2.0 mL	up to 2.0 mL

Absorbance was measured at λ=578 nm after 900 s incubation at room temperature. Results are presented as RSA.

2.3. DPPH assay.

For each concentration, the absorbance of two types of samples was measured at λ=517 nm – "control" and "sample". The total volume of the cuvette was 2.00 mL. Compositions are represented in Table 2.

Table 2. Sample compositions for the DPPH assay.

Reagent	Control	Sample
BNP02/ BNP04	none	200 μL
DPPH	1800 μL	1800 μL
Ethanol	none	none
Bi-distilled water	200 μL	none

The HAT activity of the substances was presented as RSA. Absorbance was measured 900 seconds after incubation of the reaction mixture at room temperature.

2.4. ABTS assay.

For each concentration, the absorbance of two types of samples was measured at λ=660 nm – "control" and "sample". Total cuvette volume was 1.00 mL. Compositions are presented in Table 3.

Table 3. Sample compositions for the ABTS assay.

Reagent	Control	Sample
BNP02/ BNP04	none	100 μL
R1	860 μL	860 μL
R2	40 μL	40 μL
Bi-distilled water	100 μL	none

The participation in electron-exchange reactions was presented as RSA and calculated using the same formula as previous tests. Absorbance was measured after 900 seconds of incubation at room temperature.

3. Results and Discussion

3.1. 2-Deoxyribose degradation assay.

The results from the 2-deoxyribose degradation assay are presented in Figure 2 (higher score represents greater radical scavenging).

Both substances suppress the degradation of 2-deoxyribose in a concentration-dependent manner. Even at the lowest tested molarity, 1×10^{-6} M, both compounds suppress 2-deoxyribose degradation (RSA= 8.49±1,25 % and 12.51±5,03 % for BNP04 and BNP02, respectively). They manifest statistically the same activity over the entire spectrum of tested concentrations. At 3×10^{-5} M, BNP04 and BNP02 have RSA= 62.98±1.08 % and 60.42±2.66 % respectively. The sodium salt exhibits an even stronger effect at 1×10^{-4} M, RSA= 80.47±0.68 %.

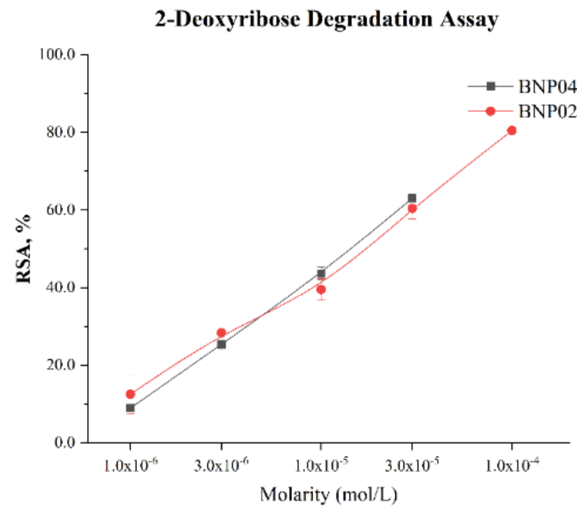

Figure 2. Impact of BNP02 and BNP04 on the degradation of 2-deoxyribose, caused by UV-induced water radiolysis. Data=Mean ±StDev, $p<0.05$.

3.2. Fenton reaction assay.

Results from the Fenton reaction assay in the presence of MTT are presented in Figure 3 (higher results correspond to a stronger scavenging effect).

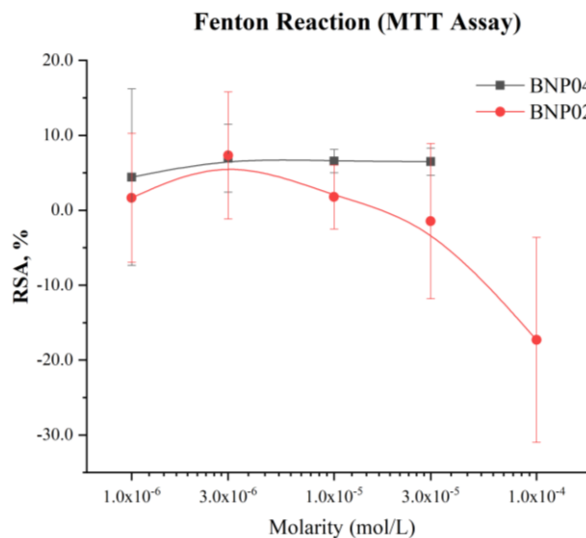

Figure 3. Impact of BNP02 and BNP04 on MTT-formazan transformation, induced by Fenton reaction-generated hydroxyl radicals. Data=Mean ±StDev, $p<0.05$.

Within the tested concentration range (1×10^{-6} M to 3×10^{-5} M), BNP04 behaves as a mild scavenger, RSA varying between 4.43±11.78 % and 6.49±1.81 % as molarities increase. On the contrary, the sodium salt BNP02 generally does not manifest statistically significant scavenging activity. Moreover, at 1×10^{-4} M, it even increases MTT-formazan transformation, seemingly acting as a mild pro-oxidant (RSA= -17.30±13.67 %).

3.3. DPPH assay.

The results from the DPPH assay are presented in Figure 4 (higher score represents greater radical scavenging).

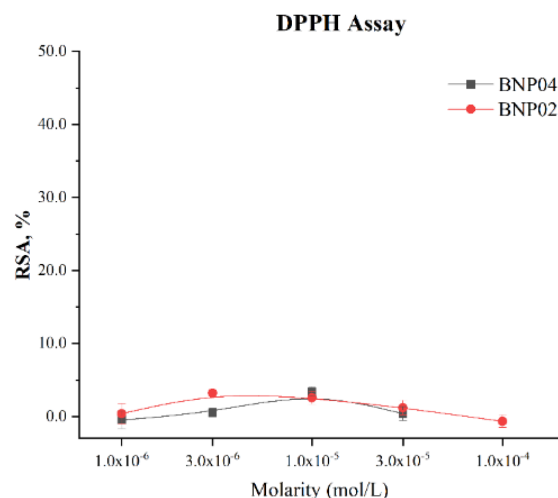

Figure 4: Impact of BNP02 and BNP04 on $^{\bullet}$DPPH. Data=Mean ±StDev, $p<0.05$.

Both compounds manifested a very mild ability to exchange hydrogen with the DPPH stable radical with RSA, never exceeding about 3%. DPPH$^{\bullet}$ has a large molecule, and its unpaired electrons may be sterically hindered from interacting with the active hydrogen atoms in BNP02 and BNP04, manifesting as seemingly low hydrogen-donating activity. A similar ligand (with a chlorine atom instead of the methoxy group) behaved as a stronger scavenger in the same model system [23]. The observed difference could be due to differences in electron density, resulting from the electron-donating methoxy group in BNP02/04 on one hand and their previously reported counterparts, bearing an electron-withdrawing chlorine atom instead.

3.4. ABTS assay.

The results from the ABTS assay are presented in Figure 5 (a higher score represents greater radical scavenging).

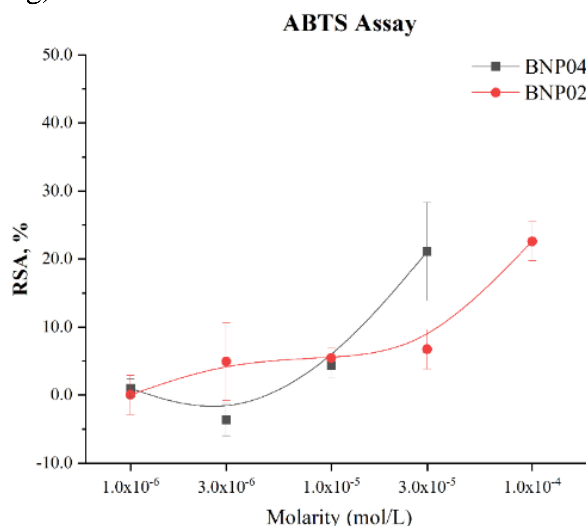

Figure 5. Impact of BNP02 and BNP04 on ABTS$^{\bullet+}$. Data=Mean ±StDev, $p<0.05$.

At the highest tested concentrations, both compounds interacted with ABTS$^{\bullet+}$ to a limited extent, the activity of BNP04 being higher at 3×10^{-5} M (RSA= 21.08±7.23 % compared to 6.73±2.90 % for BNP02). The scavenging activity of BNP02 at 1×10^{-4} M (RSA= 22.59±2.86 %) was statistically the same as that of its conjugate acid BNP04 at 3×10^{-5} M. The activities of both seem to decrease in a concentration-dependent manner to near zero within the limits of the experimental error at 1×10^{-5} M. Unlike with the DPPH assay, the chlorine-bearing

counterparts of BNP02 and BNP04 manifested significantly weaker activity, which may be due to the lack of electron-donating methoxy substituent at the benzene ring.

4. Conclusions

Based on the aforementioned experimental data, a number of conclusions can be drawn. Both BNP02 and its conjugate acid BNP04 are *in-vitro* scavengers of free radicals derived by UV radiolysis of water in the presence of 2-deoxyribose. Their activity is significant at the highest tested molarities and decreases in a concentration-dependent manner. Both compounds manifest identical activity at the same concentration. The observed high suppression of 2-deoxyribose degradation is in stark contrast with results derived by the other $OH^{\bullet}$-generating model system involving the Fenton reaction. In the latter case, BNP04 seems to be inert at all molarities. BNP02 at 1×10^{-4} M seems to act as a weak pro-oxidant. Both compounds manifest a very slight, near-zero ability to participate in HAT reactions with $DPPH^{\bullet}$. As hydroxyl and hydroperoxyl radicals (small and mobile species), formed in UV-induced water radiolysis are usually scavenged by way of hydrogen donation [24], the authors propose that the observed low activity may be due to steric hindrance [25], resulting from the relatively large size of $DPPH^{\bullet}$. Both compounds interact with $ABTS^{\bullet+}$ - BNP04, which seems to be a better scavenger of the stable radical at 3×10^{-5} M compared to its conjugate acid. The activity decreases in a concentration-dependent manner to practically zero at 1×10^{-5} M for both compounds. Similar to $DPPH^{\bullet}$, steric effects may play a role in the observed activity [26]. The triazole derivative BNP02 is associated with a significant reduction of UV-induced degradation in the *in vitro* 2-deoxyribose model system. As a ligand in a novel, biologically active lanthanide complex, it could serve as an antioxidant "shell", protecting healthy tissues from the action of the lanthanide coordination center until the target site for the physiological action is reached. The authors' plan for future research is to synthesize such complexes with triazole-bearing ligands and investigate their antioxidant properties in light of the findings of the present paper.

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Declared none.

Conflicts of Interest

The authors declare no conflict of interest.

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