*Short communication*

# In *Vitro* Antioxidant Activity of Two 6-(propan-2-yl)-4-methyl-morpholine-2,5-diones

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## Abstract

*In vitro* antioxidant activity of two cyclodidepsipeptides, 3-(2-methylpropyl)-6-(propan-2-yl)-4-methyl-morpholine-2,5-dione and 3,6-di(propan-2-yl)-4-methyl-morpholine-2,5-dione, was investigated. Our data indicate moderate antioxidant potentials of the two studied cyclodepsipeptides. A high correlation between 2,2-diphenyl-1-picrylhydrazyl (DPPH)-radical scavenging capacity and total reducing power were found. According to the density functional theory (DFT) calculations, the most probable mechanism of antioxidant action is hydrogen atom abstraction from the activated C-H group at 3-position in the morpholinedione ring. To the best of our knowledge this is the first report about the antioxidant properties of morpholine-2,5-diones derivatives.

**Keywords:** Cyclodidepsipeptides, antioxidant activity, reaction mechanism

## 1. Introduction

Cyclodepsipeptides display a variety of biological effects, such as immunosuppressant, antibiotic, antifungal, anti-inflammatory or antitumor activities.[1] Members of this class of potential drugs may also serve as leading compounds for more pharmacologically potent and toxicologically safe derivatives. Some of these natural products and (semi)synthetic derivatives have already been evaluated in clinical trials.[2]

Among the large family of cyclodepsipeptides, the simplest members are the cyclodidepsipeptides which have an ester group and an amide group in the same 6-membered ring. Two cyclodidepsipeptides, 3-(2-methylpropyl)-6-(propan-2-yl)-4-methyl-morpholine-2,5-dione (**1**) and 3,6-di(propan-2-yl)-4-methyl-morpholine-2,5-dione (**2**) (Figure 1.), were found for the first time in the natural products as potential precursors of enniatin B in the pathogenic fungi *Fusarium sporotrichioides*, isolated from the stem of fresh *Hypericum barbatum* Jacq. For identification and confirmation, those compounds were synthesized and studied by density functional theory calculations and infrared spectroscopy.[3]

Only one report concerning biological activity of the two cyclodepsipeptides has been published, treating possible immunomodulatory effect and antimicrobial activity of **1** and **2.** The citotoxic effect of **1** and **2** on rat thymocytes demonstrated that increasing concentrations (0.1, 1, 10 μg/well) of **1** and **2** to cell culture, showed no significant effect on thymocytes toxicity. The minimal inhibitory concentration (MIC) of **1** and **2** against two Gram-positive and three Gram-negative bacteria ranged between 2 and 25 mg/ml.[4]

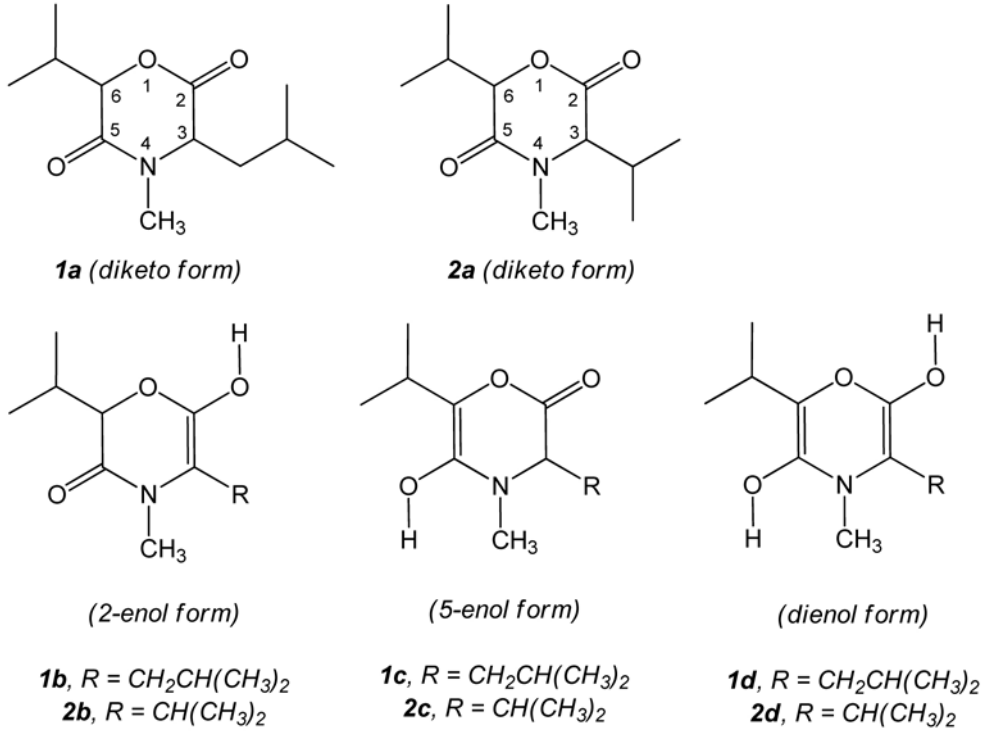


**Figure 1.** Chemical structures of the compounds under study and their tautomeric forms.

In the present study, antioxidant activity of the two synthesized cyclodidepsipeptides was investigated applying two assays, DPPH-radical scavenging capacity and total reducing power. An attempt to correlate the examined pharmacological effects with the structure of the studied compounds was undertaken, and a possible mechanism of antioxidant action was proposed. To the best of our knowledge, this is the first report on the antioxidant properties of morpholine-2,5-diones derivatives.

## 2. Experimental

The synthesis of the compounds, 3-(2-methylpropyl)-6-(propan-2-yl)-4-methyl-morpholine-2,5-dione and 3,6-di(propan-2-yl)-4-methyl-morpholine-2,5-dione, was described in our recent study.[3] Antioxidant activity of the two compounds was examined by DPPH radical scavenging assay and Fe(III) to Fe(II) reducing power assay.

**DPPH$^{\bullet}$ radical scavenging capacity.** Ten μL of each solution (both compounds and reference standard BHT) was mixed with 90 μmol/L DPPH$^{\bullet}$ in methanol (1.0 mL) and made up with methanol to a final volume of 4.0 mL. The mixtures were shaken, incubated in dark for 60 min at room temperature and the absorbance of resulting solution was measured at 517 nm applying Perkin-Elmer Lambda 15 UV-VIS spectrophotometer. The DPPH$^{\bullet}$ scavenging activity was expressed by radical scavenging capacity using the following equation:

$$\text{DPPH}^{\bullet}\ \text{RSC}(\%) = 100\ (A_0 - A_1/A_1)$$

where $A_0$ was the absorbance of the control reaction (full reaction, without the tested solution or BHT) and $A_1$ was the absorbance in the presence of the sample.

**Total reducing power assay.** Each solution (10 μL) was mixed with 1% $K_3[Fe(CN)_6]$ (1 mL) and phosphate buffer (1 mL, 0.2 mol/L, pH 6.6). These mixtures were incubated at 323 K for 30 min, then 10% trichloroacetic acid (1 mL) was added and mixtures were centrifuged at 3000 rpm for 10 min. Finally, the supernatant fractions (1 mL) were mixed with distilled water (1 mL) and 0.1% $FeCl_3$ (0.2 mL). The absorbances of resulting solutions were measured at 700 nm. Fe(III) to Fe(II) reducing power was calculated using the following equation:

$$\text{AEAC} = C_A(A_S/A_A)$$

where $C_A$– final concentration of ascorbic acid in μg/mL, $A_S$– absorbance of the sample, $A_A$– absorbance of ascorbic acid. Reducing power was expressed in relation to the reducing power of ascorbic acid as a positive control.[5]

All experiments were done in triplicate. The experimental results were expressed as mean±standard deviation (SD). Correlation coefficients to determine the rela-

tionship between two different antioxidant assays were calculated using MS Excel software (CORREL statistical function).

## 2. 1. Calculation

The geometries of all possible isomers of the studied compounds, radicals, radical cations were fully optimized by application of the UB3LYP functional in conjunction with the 6–311++G** basis set. The optimized structures were further characterized by analytic computations of harmonic vibrational frequencies at the same level. Dissociation enthalpy (BDE), ionization potential (IP), proton dissociation enthalpy (PDE), proton affinity (PA), and electron transfer enthalpy (ETE) were calculated according the equations given by Klein *et al*.[6] Obtained total energy of the hydrogen atom, –0.502257 Hartree, was used in the BDE calculations. The calculated enthalpy of proton, H(H+), is 6.197 kJ/mol; the enthalpy of electron, H(e–), is 3.145 kJ/mol. All reaction enthalpies were calculated for 298 K.

# 3. Results and Discussion

DPPH scavenging activities of both tested compounds, 3-(2-methylpropyl)-6-(propan-2-yl)-4-methyl-morpholine-2,5-dione (compound **1**) and 3,6-di(propan-2-yl)-4-methyl-morpholine-2,5-dione (compound **2**), in highest concentrations (10 μg/ml), compared to the standard BHT (butylated hydroxytoluene) solution (30 μg/ml), were estimated as very high (Table I), considering the concentration ratio of both compounds and referent standard.

**Table 1.** DPPH-radical scavenging capacity and total reducing power of compounds **1**, **2** and reference compound, BHT

| Compound | Concentration (μg/ml) | DPPH-radical scavenging capacity (%) | Total reducing power (ascorbic acid equivalents, μg /ml) |
|---|---|---|---|
| 1 | 10 | 18.67±0.12 | 159.51±1.65 |
| 1 | 1 | 0.80±0,05 | 55.21±0.56 |
| 1 | 0.1 | 0.60±0.05 | 36.81±0.23 |
| 2 | 10 | 19.28±0.17 | 73.62±0.71 |
| 2 | 1 | 1.81±0.08 | 30.67±0.22 |
| 2 | 0.1 | 1.20±0.06 | 6.13±0.09 |
| BHT | 30 | 38.42±0.75 | – |

Values expressed are means ± SD of three parallel measurements

Total reducing powers of the two compounds, comparing to ascorbic acid as a reference, were also found to be high and concentration dependent. Compound **1** showed higher reducing power in comparison with compound **2**, which may be conditioned by steric differences.

Correlation coefficients between DPPH-radical scavenging capacity and total reducing power were 0.992 and 0.944 for compounds **1** and **2**, respectively, which implies very good agreement of data, achieved by two different antioxidant assays.

Oxidation reactions can produce free radicals which, may lead to damage or even cell death. In general, antioxidants remove free radicals and inhibit other oxidation reactions, often oxidizing themselves, playing in that way role of reducing agents.[7] In order to explain antioxidant action of naturally occurring and synthetic antioxidants two mechanisms are generally accepted – hydrogen atom transfer (HAT mechanism) and single-electron transfer (SET mechanism).[8–10] More recently a third one has been discussed – sequential proton loss electron transfer (SPLET).[6, 11] Based on calculation of the reaction enthalpies related to the three antioxidant action mechanisms, it is possible to suggest the most probable mechanism of action of a particular group of compounds.[6] For this reason, next step of the present study was computational estimation of the ability of compounds **1** and **2** to be oxidized according to above-mentioned mechanisms. Different molecular structures of compounds **1** and **2**, able to form radicals and act as reducing agents were examined (Figure 1) using the density functional theory (DFT) employing the UB3LYP functional and the 6–311++G** basis set. Most often, the antioxidant activity is due to the presence of hydroxyl groups, including such formed as a result of keto-enol tautomerisation. Rezk *et al.*,[12] previously proposed mechanism of antioxidant activity of 2,6-dihydroxyacetophenone whose phenol groups and tautomerization process are of crucial importance for its antioxidant activity. Abstraction of hydrogen atom from activated C-H groups has also been proposed by some authors as responsible for antioxidant activity.[13,14] Compounds **1** and **2** could undergo tautomerization and form 2- and 5-enol („**b**„ and „**c**„) or dienol structures („**d**„). The calculated ZPVE-corrected total energies (Table II.) indicate the diketo form „**a**„ as the most stable. According to Minkin *et al*.[15] prototropic conversions are probable in case when the energy differences between the initial and the final structure do not exceed 25 kJ/mol, with activation barrier not higher than 105 kJ/mol. The energy differences for all enol forms „**b**–**d**„ of compounds **1** and **2** are much larger, as it could be seen in Table II, and convinces that only keto form should be expected to exist in real systems.

It is in agreement with the reported experimental NMR and IR data[3] for **1** and **2** showing that both compounds are present in diketo form in solid state and chloroform. The possibility of prototropic tautomerism of a related compound, 6-(propan-2-yl)-3-methyl-morpholine-2,5-dione, was recently studied in solution and in this case again no evidences for enol formation were found neither in polar nor in nonpolar medium.[16]

**Table 2.** Calculated total energies $E_{corr}$ and relative energies of studied species.

| Species | $E_{tot}$, (Hartree) | ΔE, kJ/mol |
|---|---|---|
| 1a | –750.266255 | 0.00 |
| 1b | –750.242225 | 63.09 |
| 1c | –750.225798 | 106.22 |
| 1d | –750.193992 | 189.73 |
| 2a | –710.962474 | 0.00 |
| 2b | –710.943003 | 51.12 |
| 2c | –710.930147 | 84.87 |
| 2d | –710.895483 | 75.88 |

The reaction enthalpies related to the formation of radicals by compounds **1a** and **2a** via HAT, SET and SPLET were calculated in order to outline the most probable mechanism of the antioxidant action. The results are summarized in Table III. The calculated C–H bond dissociation enthalpies (BDEs) fall within the range 294–306 kJ/mol. They are comparable to those reported for tocopherol family[6] and catechins,[13] and lower than the BDE of phenol.[17] Hence the computational estimation supports the high antioxidant activity of compounds **1** and **2** demonstrated by the experiment. IPs could be used to judge on the contribution of SET reaction pathway. It is known that if IPs drop to *ca.* 167 kJ/mol below phenol, the SET mechanism gain importance in solution.[8] In the present case, none of the studied structures is characterized by IP value lower than the IP of phenol, thus excluding the SET as possible mechanism of action. The data in Table III reveal that the PAs of **1a** and **2a** radicals are significantly higher than the corresponding BDEs. From the thermodynamic point of view, it implies HAT as the most probable mechanism of action in gas phase. On the other side, it was experimentally demonstrated that antioxidants could react with DPPH and other electron deficient radicals by two different and nonexclusive mechanisms, HAT and SPLET, in ionization supporting solvents such as water, DMSO or ethanol.[10] Therefore, domination of SPLET mechanism can be anticipated in solution.

**Table 3.** DFT bond dissociation enthalpy (BDE), ionization potential (IP), proton dissociation enthalpy (PDE), proton affinity (PA), and electron transfer enthalpy (ETE) values of the studied species in kJ/mol.

| Species | BDE [a] | IP [a] | PDE [a] | PA [a] | ETE [a] |
|---|---|---|---|---|---|
| 1a | 306.47 | 824.66 | 782.89 | 1439.76 | 167.80 |
| 2a | 294.07 | 818.66 | 776.50 | 1427.60 | 167.56 |

[a] Calculated according to Musialik[11]
[b] IP of phenol is calculated as 807 kJ/mol in the same basis set. [17]

# 4. Conclusions

The data resulted from these screening experiments indicate that compounds **1** and **2** show excellent antioxidant activity. According to the DFT calculations, the most probable mechanism of antioxidant action is hydrogen atom abstraction from the activated C–H group at 3-position in the morpholinedione ring. Recently we have shown that two studied cyclodidepsipeptides do not induce the toxicity and mitochondrial membrane potential decrease in rat thymocytes and, for the first time for this group of compounds, do not trigger the significant intracellular reactive oxygen species production and exhibited antibacterial activity. On the other hand, higher concentrations of two studied cyclodidepsipeptides were able to stimulate proliferative activity of thymocytes, with mechanisms not solved yet, indicating potential stimulatory effect on the cells of the immune system.[4] In this way, compounds **1** and **2** may give a promise to be used as antioxidants and dietary supplements.

# 5. Acknowledgements

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## Povzetek

Proučevali smo antioksidativno in antiholinesterazno aktivnost dveh ciklodidepsipeptidov, 3-(2-metilpropil)-6-(propan-2-il)-4-metil-morfolino-2,5-diona in 3,6-di(propan-2-il)-4-metil-morfolino-2,5-diona. Naši podatki nakazujejo na visok antioksidativni potencial obeh proučevanih ciklodepsipeptidov. Opazili smo visoko korelacijo med odstranjevalno sposobnostjo 2,2-difenil-1-pikrilhidrazil (DPPH)-radikala in celokupno sposobnostjo redukcije. Glede na izračune po Teoriji funkcionalne gostote (DFT) je najbolj verjetni mehanizem antioksidativne aktivnosti odvzem vodikovega atoma z aktivirane skupine C-H na položaju 3 morfolindionovega obroča. Preiskovane spojine niso pokazale aktivnosti proti holinesterazi v združenih človeških serumih, opazili pa smo nizko aktivacijo encima. Po naših podatkih je to prvo poročilo o antioksidativnoh lastnostih in holinesterazni inhibiciji derivatov morfolino-2,5-dionov.