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Convenient synthesis of quinocetone metabolites: Characterization, theoretical investigation, and cytotoxicity study

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HIGHLIGHTS

- Four main metabolites of quinocetone were conveniently synthesized and fully characterized.
- Theoretical N–O bond dissociation enthalpies and the octanol-water partition coefficient (K_{ow}) were estimated.
- The results from MTT assay have showed that quinocetone and its metabolites have cytotoxicity.

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ABSTRACT

Quinocetone (3-methyl-2-quinoxalinbenzenevinylketo-1,4-dioxide; QCT) is a new promising antimicrobial growth promoter for quinoxalines. The identification of the major metabolites of QCT has resulted in a number of studies regarding its metabolic pathway. However, little is known about the systematic synthesis, characterization, and simultaneous determination of its metabolites. To obtain system data for the four main metabolites of QCT, a convenient synthesis of these compounds was performed. All synthesized compounds were characterized by infrared spectroscopy, nuclear magnetic resonance, and high-resolution mass spectroscopy. The theoretical N—O bond dissociation enthalpies (BDEs) and octanol–water partition coefficient (K_{ow}) were estimated. A cytotoxicity assay for these compounds in hepatocytes isolated from rats was proposed, and the cytotoxicity results were evaluated based on the calculated N—O BDEs.

1. Introduction

Quinocetone (3-methyl-2-quinoxalinbenzenevinylketo-1,4-dioxide; QCT) belongs to the family of quinoxaline-1,4-dioxides (QdNOs), a class of bioactive compounds that exhibit notable antibacterial, antiviral, and antifungal properties [1]. Since the 1960s, several quinoxaline-1,4-dioxides have been developed as veterinary drugs to promote growth, prevent dysentery, and inhibit bacterial enteritis in farm animals [2]. However, two well-known QdNOs drugs, carbadox (CBX) and olaquindox (OLA), were banned in 1999 due to toxicity and food safety concerns [3]. As a new promising synthetic veterinary drug, QCT retains the advantages of QdNOs and can inhibit bacteria such as *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella*. The lower toxicity level of QCT than CBX and OLA has ensured its availability in the Chinese market as a pharmaceutical agent for swine, poultry, and aquatic animals since 2003 [4]. The majority of pharmacokinetic studies and toxicology research on veterinary chemicals have focused on the metabolites of QdNOs. A number of studies have drawn similar conclusion that the toxicities of QdNOs are closely associated with their metabolites [5–8]. Our previous study has revealed that QCT is rapidly metabolized in the liver and kidneys of pigs, thereby producing several desoxy and reduction metabolites [9].

These compounds are found as residues in edible swine tissue and can be traced through the gastrointestinal tract of pigs. However, there are limited characterization data for the four main metabolites, namely, 1,4-bisdesoxyquinocetone (1,4-BDQ), 1-desoxyquinocetone (1-DQ), 4-desoxyquinocetone (4-DQ), and 3-methyl-2-styrenol-quinoxaline (MSQ). Most available toxicity studies on QdNOs drugs have focused on the parental drugs rather than their metabolites. Nevertheless, these drugs are rapidly metabolized into various kinds of metabolites in vivo, and a few of the reacting intermediates or final products have been identified and quantified. To the best of our knowledge, there is no available information regarding the cytotoxicity of 1,4-BDQ, 1-DQ, 4-DQ, and MSQ, except for several articles focusing only on QCT [10].

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There are numerous methods for the selective monodeoxygenation and reduction of certain QdNOs [11–13]. However, there are only a few procedures for the synthesis of the metabolites of QdNO veterinary drugs. Recently, Li et al. [14] have prepared 1,4-BDQ, 1-DQ, and 4-DQ from the parent compound QCT using different selective reagents. Our group has also reported procedures for the preparation of metabolites from mequindox (MEQ), another form of QdNO veterinary drug [15,16]. The current study reports a simple one-step procedure based on the structural similarities between QCT and MEQ to synthesize 1,4-BDQ, 1-DQ, and 4-DQ from the corresponding metabolites of MEQ. MSQ was also subsequently synthesized from 1,4-BDQ.

Oxygenated species, including heterocycles containing one or more N—O bonds, have been ordered to establish a reactivity scale based on their abilities to transfer oxygen atoms in several biochemical conversions [17]. A theoretical approach for understanding the toxicity of QdNOs is at an early stage; however, some steps that depend on N—O bond dissociation enthalpies (BDEs) have been established. The N—O bond may also lower the octanol–water partition coefficient (K_{ow}), which can influence the sorption in vivo or sediments for agricultural applications [8]. Therefore, studies on the N—O BDEs of QdNOs and K_{ow} are important in the assessment of drugs. In the present study, calculations of BDEs and K_{ow} using accurate density functional theory (DFT) and other computational methods combined with cytotoxicity assay are crucial for determining pharmacological importance and biologic activity.

In the current study, a convenient synthesis and characterization, including the determination of the K_{ow} values of the four main metabolites of QCT, were described. The first, second, and mean N—O BDEs for QCT were predicted based on DFT calculations. Toxico-kinetic and cytotoxicity assays in hepatocytes isolated from rats were proposed. The results were evaluated and associated with each calculated N—O BDE. This systematic research on QCT and its metabolites provides useful information on the food safety evaluation of QCT.

2. Materials and methods

2.1. Materials

All reagents and solvents used in the synthesis were analytical grade and purchased from the Beijing Chemical Reagent Company. MEQ (purity 98%) and QCT (purity 98%) were provided by the College of Veterinary Medicine, Huazhong Agricultural University (Wuhan, China). 1,4-bisdesoxymequindox (1,4-BDM, purity ⩾ 99%), 1-desoxymaquindox (1-DM, purity ⩾ 99%) and 4-desoxymaquindox (4-DM, purity ⩾ 99%) were synthesized according to our published protocols [15,16]. 3-(4,5-Dimethylthia-zol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), N-(2-hydroxyethylpi-perazine)-N′-(2-ethane sulfonic acid) (HEPES), collagenase, Dulbecco's modified Eagle medium (DMEM), fetal calf serum (FCS), insulin, and collagen were purchased from Sigma Chemical Company (St. Louis, MO). Antibiotics (penicillin–streptomycin mixtures) were purchased from GIBCO Company (USA). All other chemicals and solvents used in the extraction and cleanup procedures were analytical grade and purchased from commercial sources.

Adult male Sprague–Dawley rates (200–250 g) which were fed with a standard diet and fasted 12 h before the experiment were provided by the Experimental Animal Research Institute of China Agricultural University.

2.2. Instruments

Melting points were measured with a WRX-4 micro melting point apparatus (Shanghai Yice Co., China) and were uncorrected. ^{1}H and ^{13}C NMR spectra were measured on a Bruker DSX-300 instrument at 100.6 and 40.6 MHz, respectively, in $CDCl_3$ with TMS as the internal standard. The chemical shifts are provided in δ (ppm). The UV and IR spectra were recorded on a Pgeneral T6 new century UV–VIS spectrophotometer and a PerkinElmer FT–IR Spectrum 100 spectrometer, respectively. High resolution mass spectrometry (HRMS) were performed on an ACQUITYUPLC™ BEH C18 column (50 mm × 2.1 mm i.d., 1.7 μm particle size; Waters Co., Milford, MA, USA) and recorded with a UPLC/ESI-QTOF-MS system (Waters, Manchester, UK) controlled by MassLynx™ software.

2.3. Methods

Unless otherwise noted, all operations including the synthesis and analysis were performed in the absence of light or under very low light conditions because of the lability to light of the target compounds. The procedures for infrared (IR) spectroscopy, nuclear magnetic resonance (NMR) imaging, corresponding absorption spectrums and reverse-phase HPLC analyzes can be found in the Supplementary Materials.

2.3.1. Preparation of 1,4-BDQ

The synthesis of 1,4-BDQ was performed at 45 °C under continuous stirring of a mixture containing 60 mL of ethanol, 5.83 g (55.0 mmol) of benzaldehyde, 7.44 g (40.0 mmol) of 1,4-BDM, and 1.04 g (26 mmol) of sodium hydroxide. The solution was filtered in 95% ethanol after 1 h and afforded 5.95 g (21.7 mmol) of 1,4-BDQ as a yellow solid. m.p. 144.6–147.0 °C. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 3.01 (3, s, CH_3), 7.45–7.88 (m, Ar—H, CH═CH, 9), 8.05–8.17 (m, C_8—H, C_5—H, 2); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 24.029, 123.399, 127.830, 128.232, 128.533, 128.809, 128.945, 129.579, 129.749, 130.807, 131.737, 134.843, 139.737, 142.492, 158.588, 148.640, 153.577; IR (KBr) v: 649, 776, 937, 1123, 1190, 1361, 1482, 1694 cm^{-1}; MS (ESI) m/z (%): 275.11 $[M + H]^+$. HRMS (ESI) calcd for $C_{18}H_{15}N_2O$ $[M + H]^+$ 275.3301, found 275.1159.

2.3.2. Preparation of 1-DQ and 4-DQ

First, 1-DM (8.08 g, 40.0 mmol) or 4-DM (8.08 g, 40.0 mmol) was dissolved in 100 mL of ethanol and cooled to 0 °C in an ice bath. This step was followed by the slow addition of 6.36 g (60.0 mmol) of benzaldehyde and 3.70 g (50.0 mmol) of diethylamine. After 0.5 h, the precipitate was filtered and washed with 95% ethanol to yield 8.73 g (30.1 mmol, yellow solid) of 1-DQ or 7.14 g (24.6 mmol, yellow solid) of 4-DQ. For 1-DQ: m.p. 155.0–156.3 °C. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 2.85 (3, s, CH_3), 7.26–7.86 (Ar—H, CH═CH, 9), 8.21 (m, C_8—H, 1), 8.65 (m, C_5—H, 1); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 13.9, 118.9, 123.3, 128.9, 129.0, 130.5, 130.7, 131.0, 131.5, 131.8, 134.5, 137.0, 140.4, 141.9, 150.2, 151.7, 189.9, 200.1; IR (KBr) v: 764, 989, 1343, 1573, 1596, 1670 cm^{-1}; MS (ESI) m/z (%): 291.62 $[M + H]^+$. HRMS (ESI) calcd for $C_{18}H_{15}N_2O_2$ $[M + H]^+$ 291.3291, found 291.6168. For 4-DQ: m.p. 222.0–224.6 °C. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 2.66 (3, s, CH_3), 7.13–7.88 (Ar—H, CH═CH, 9), 8.09 (m, C_8—H, 1), 8.55 (m, C_5—H, 1); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 22.28, 118.78, 125.06, 128.87, 128.99, 129.39, 129.74, 131.34, 132.28,133.92, 135.39, 137.25, 144.37, 146.14, 153.42, 188.40; IR (KBr) v: 765, 1034, 1128, 1348, 1405, 1483, 1605, 1669 cm^{-1}; MS (ESI) m/z (%): 291.10 $[M + H]^+$. HRMS (ESI) calcd for $C_{18}H_{15}N_2O_2$ $[M + H]^+$ 291.3291, found 291.1026.

2.3.3. Preparation of MSQ

MSQ was prepared from 1,4-BDQ. KBH_4 (aq, 0.3 mol/L) was added dropwise to a solution of 1,4-BDQ (4.14 g, 15 mmol) in anhydrous alcohol at a temperature of approximately 30 °C. The reactions were monitored using TLC (thin-layer chromatography,

ethyl acetate/petroleum ether mixtures, 1:1) with pre-coated silica gel aluminum plates containing a fluorescent indicator. The reactions were stopped when there was only one spot visible by TLC. Most of the solvent was subsequently removed using a rotary evaporator. Water (30 ml) was added, and the mixture was washed three times with chloroform (30 mL at a time). The organic phases were combined and dried with anhydrous sodium sulfate. Then the Crude MSQ was obtained by removing the solvent on a rotary evaporator. The crude product was dissolved in warm diethylether (80 mL) and kept at 4 °C for 12 h. The resulting white crystals were separated from the mixture. The recrystallization was repeated three times using diethyl ether to afford pure MSQ (3.04 g, 11 mol, pale yellow solid). For MSQ: m.p. 84.0–85.1 °C. ^{1}H NMR (DMSO, 300 MHz) δ 2.86 (3, s, CH_3), 3.43 (1, d, -OH), 5.71 (1, m, 2-CH), 5.75 (m, C_8—H, 1), 6.10 (m, C_5—H, 1), 6.88–8.08 (Ar—H, CH=CH, 9); ^{13}C NMR (DMSO, 75 MHz) δ 22.459, 73.600, 126.610, 127.739, 128.405, 128.507, 128.655, 128.750, 129.135, 129.625, 129.820, 130.155, 130.379, 136.644, 140.872, 142.150, 153.188, 156.187; IR (KBr) v: 692, 757, 968, 1080, 1449, 1486, 1565, 1596 cm^{-1}; MS (ESI) m/z (%): 276.13. $[M+H]^+$. HRMS (ESI) calcd for $C_{18}H_{17}N_2O$ $[M+H]^+$ 277.1336, found 277.1341.

2.4. Theoretical study

N—O BDEs were calculated using the Gaussian 09 program [18]. The geometries of the molecules and related radical species were optimized at the B3LYP/6-31 + g(d,p) level, which has been used in numerous structure optimizations. This method is feasible because of the high accuracy requirements and practical, acceptable computational cost [19,20]. Subsequent frequency calculations at the same level verified that these optimized structures were the global minimum without imaginary frequencies and provided thermal data. Single-point energies for the optimized structures of the molecules and radicals were refined and computed with the more flexible B3LYP/6-311 + g(2df,2pd) level. The energies obtained from the extended basis set calculations were used to estimate the N—O BDEs. The gas-phase mean N—O molar BDEs for MEQ were used as a reference to validate the theoretical method. The theoretical K_{ow} values for QCT, 1,4-BDQ, 1-DQ, 4-DQ, and MSQ were calculated based on the constants assigned to the various functional groups using the ChemAxon computer program [21].

2.5. Cytotoxicity study

The hepatocytes were isolated by the two-step collagenase perfusion method. In the first step, about 100 g of liver was washed in 500 mL of 20 mM HEPES solution without calcium to remove blood and weaken the cell–cell junctions. The hepatocytes were released by collagenase (50 mg/100 mL) perfusion for 20 min (recirculation system), centrifugation, and resuspension thrice in the second step. After isolation, the hepatocytes were suspended in DMEM containing 10% FCS, 100.0 g/mL streptomycin, 100 U/mL penicillin, and 10.0 g/mL insulin at pH 7.65. The hepatocytes were then placed into 100-mm diameter plastic dishes, pre-coated with collagen, and maintained in a 5% CO_2 incubator at 37 °C. To determine cytotoxicity, the hepatocytes were plated onto 96-well plates (10^4 cells per well). After the monolayers of cells became confluent in the 96-well plate, the rat hepatocytes were treated with different concentration ranges of QCT, 1,4-BDQ, 1-DQ, 4-DQ, and MSQ in DMEM without FCS, with no drug (control), and/or with DMSO alone (vehicle control) for 4 h. The vehicle controls were specified with a volume of DMSO corresponding to the largest volume of test compound added to the treatment dishes. Various toxicity end points were assayed in the control and drug-exposed cells after 4 h. Mitochondrial function was evaluated by measuring the degree of reduction of the tetrazolium salt MTT to formazan by mitochondrial succinic dehydrogenase.

3. Results and discussion

3.1. Synthesis of QCT metabolites

QCT aromatic N-reduced metabolites including 1,4-BDQ, 1-DQ, and 4-DQ were synthesized by the aldol condensation of the corresponding metabolites of MEQ and benzaldehyde in the presence of sodium hydroxide in ethanol solutions. Generally, when phosphorus trichloride, trimethyl phosphate, and sodium hydrosulfite were used as the reducing agents for the selective deoxygenation of quinoxaline 1,4-dioxides, the reaction produced a large amount of byproducts, and the product was hard to purify. In contrast, the reactions in the current study proceeded very efficiently, resulting in good yields and operational simplicity in the separation of the products. Compared with the selective deoxygenation of quinocetone, the convenient and specific synthesis of several QCT metabolites can improve research on the metabolism of the veterinary drug. For the synthesis of MSQ, the hydroxide ion (ethanol potassium hydroxide) reacted rapidly with 1,4-BDM to yield 1-(3-methylquinoxalin-2-yl)-3-phenylprop-2-en-1-ol, which was easily recrystallized from an ether solution. The synthesized 1,4-BDQ, 1-DQ, 4-DQ, and MSQ were shown to be ⩾99% pure by HPLC–UV. The preparation of these compounds is outlined in Scheme 1.

3.2. Theoretical calculation results

The N—O BDEs were calculated from the enthalpy change of the following hemolytic bond dissociation reaction:

$$R—N—O(g) \rightarrow R—N^{\cdot}(g) + O^{\cdot}(g) \quad (1)$$

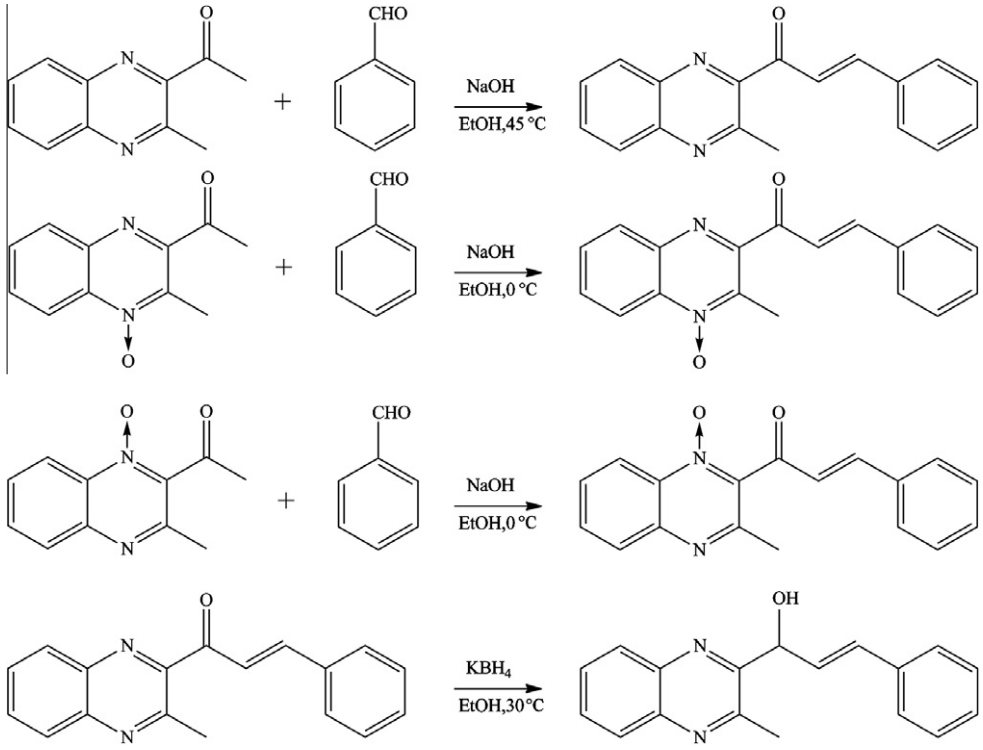
The mean N—O BDE of QCT was half of the enthalpy of the following reaction:

$$O—N—R—N—O(g) \rightarrow N—R—N^{\cdot}(g) + 2O^{\cdot}(g) \quad (2)$$

The bond dissociation energy of the N—O bond was computed from the heats of formation at 298.15 K of the species involved in the dissociation, i.e.:

$$E_{BDE} = \Delta_f H^{\circ}_{298.15,R—N} + \Delta_f H^{\circ}_{298.15,O} - \Delta_f H^{\circ}_{298.15,R—N—O} \quad (3)$$

To evaluate the accuracy of the B3LYP/6-311+g(2df,2pd) level, the experimental data for the mean N—O BDE of MEQ (251.6 ± 4.2 kJ mol^{-1}) was used as a Ref. [22]. In our calculation, the theoretical value for the mean N—O BDE of MEQ was 253.4 kJ mol^{-1}, and the difference between the theoretical and experimental values was very small. Therefore, the proposed method was suitable for calculating the N—O BDEs of QdNO derivatives. For QCT, which also has two different N—O bonds due to different chemical environments, these bonds in QCT were expected to exhibit different dissociation energies. Consequently, the N—O BDE can be described according to the first, second, total, and mean N—O BDEs. The first N—O BDE was the energy required to break the weakest bond in the di-N-oxide compound to yield the corresponding N-oxide. The second N—O BDE was the energy required to break the bond in the N-oxide compound to yield the parent quinoxaline. The total and mean N—O BDEs were the sum and mean of the former two dissociation enthalpies, respectively. The full results for the computed BDEs for QCT are schematically shown in Fig. 1. The dissociation of the 4-N—O bond, which is closer to the methyl group, occurred more easily and yielded a BDE



Scheme 1. Synthesis of 1,4-BDQ, 1-DQ, 4-DQ and MSQ.

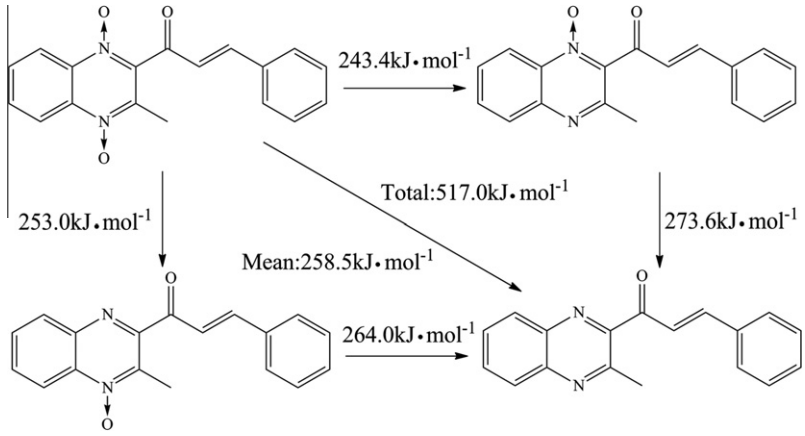


Fig. 1. First, second, total, and mean N—O BDEs for QCT were computed at the B3LYP/6-311 + g(2df,2pd) level of theory.

value of 243.4 kJ mol^{-1}. This BDE was determined to be the first N—O BDE of QCT. The metabolic pathway producing the desoxy metabolites were presumed to be the same.

Given the growing interest in using computational techniques for regulatory purposes, the prediction of K_{ow}, a well-studied chemical property, has been developed rapidly with numerous theoretical models. ChemAxon is one of the popular desktop chemical software programs that provides accurately predicted K_{ow} values based on a published database. Strock et al. [8] have examined the K_{ow} values of another QdNO derivative, CBX, and its associated

Table 1
Selected properties of quinocetone and its metabolites.

Compound	Molecular weight (g mol^{-1})	MP (°C)	logK_{ow}	LD_{50} (rat mg/kg b.w)
QCT	306.32	186.5–187.5	1.29	8687.32[a]
1,4-BDQ	274.32	144.6–147.0	3.55	–
1-DQ	290.32	155.0–156.3	2.76	–
4-DQ	290.32	222.0–224.6	2.08	–
MSQ	276.33	84.0–85.1	2.89	–

[a] Ref. [23].

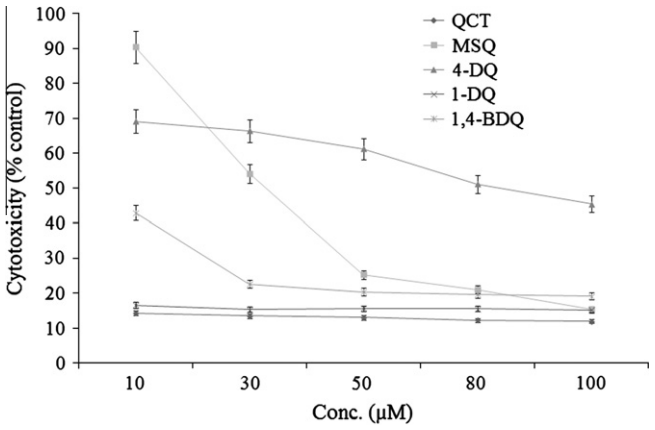


Fig. 2. Cytotoxicity results using MTT assay for each of the five chemicals. Cell cultures were exposed to the drugs for 4 h. Control value was taken as 100%. The data were presented as means ± SE from three independent experiments.

N-oxide reduced metabolites by this software, and found trends consistent with the experimental data. In addition to the selected properties of QCT and its metabolites, the K_{ow} values determined by ChemAxon are summarized in Table 1. From the log*P* plugin of ChemAxon, no significant difference at the 95% confidence level was observed in the K_{ow} measurements as a function of pH for QCT and its metabolites. The K_{ow} values were averaged for each system.

3.3. Toxicity against rat hepatocytes

The results of the MTT assay demonstrated a dose-dependent decrease in mitochondrial activity in rat hepatocytes at all studied concentrations of QCT and its metabolites (Fig. 2). The results of the MTT assays showed that QCT and 1-DQ both inhibited cell viability by 14% at 10 μmol, and displayed the most potent cytotoxicity among the studied compounds. On the contrary, treatments with MSQ and 4-DQ (10 μmol) resulted in decreased cell viability to only 10% and 30%, respectively. Combined with the N—O BDE study in this work, the results clearly elucidated the molecular mechanisms involved in drug toxicity. Based on the lower BDE of 243.4 kJ mol^{-1}, the toxicity can be mainly attributed to the cleavage of the 4-N—O bond rather than that of the 1-N—O bond. Notably, the complete elucidation of the cytotoxic mechanism of QCT is outside the scope of our current work. However, based on the MTT assay results, careful monitoring procedures are imperative for the safe use of these compounds as animal feed, and awareness of their toxic effects to animals and general consumers should be raised.

4. Conclusions

This study reported the convenient synthesis of the four main metabolites of QCT, namely, 1,4-BDQ, 1-DQ, 4-DQ, and MSQ. All synthesized compounds were characterized by IR, NMR, and HRMS. The theoretical N—O BDEs and K_{ow} were calculated by the B3LYP/6-311 + g(2df,2pd) DFT method and ChemAxon software, respectively. A cytotoxicity assay for these compounds in hepatocytes isolated from rats was also proposed. QCT and 1-DQ both displayed the most potent cytotoxicities, and the cleavage of the 4-N—O bond may be ultimately responsible for the toxicity of QdNOs. This systematic study can improve pharmacokinetic and residue studies on veterinary drugs.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molstruc.2012.04.071.

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