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# NAD(P)H: Quinone Oxidoreductase 1 inducer activity of some enaminone derivatives.

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## Abstract

**The present work reports the synthesis of some enaminone derivatives bearing the biologically active 3,4-dimethoxyphenyl (3) or 3,4,5-trimethoxyphenyl moieties (5 and 7), respectively. The trimethoxybenzene moiety has been previously reported to confer cytotoxic activity. However, we found that, at high micromolar concentrations, the new compounds have the ability to weakly induce the cytoprotective enzyme NQO1. This is most likely due to their electrophilic cyclohexenone functionality, a well-established structural feature of NQO1 inducers. The structure of the newly synthesized compounds was confirmed on the basis of elemental analyses, IR, $^{1}$H-NMR, $^{13}$C-NMR spectra.**

**Keywords**: Synthesis, enaminones, NQO1, electrophilicity, cytoprotection

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## Introduction

Enaminones are a group of organic compounds carrying the conjugated system N- C= C- C= O. The literature has reported information about the chemistry of enaminones, their physicochemical properties and biological activities [1-4]. In addition, cyclohex-2-enone has a wide range of biological activities such as anticancer [5] and antimicrobial activities [6]. On the other hand, enaminones have been extensively used as key intermediates in organic synthesis [7-12] and the chemistry of these compounds has been reviewed [13]. In particular they have been employed as synthons of a wide variety of biologically active heterocyclic compounds [14], as pharmaceutical agents with anticancer [15], antibacterial [16], anti-inflammatory [17] and other therapeutic activities [18-20]. During the past decade we have been involved in a program aimed at exploring the potential of enaminone as building blocks for heteroaromatics [21], and have successfully synthesized quinoline derivatives[7-12] utilizing enaminones as starting materials. Based on the above information and as a continuation of previous work on anticancer agents [22-27], we report the synthesis of some new enaminone derivatives.

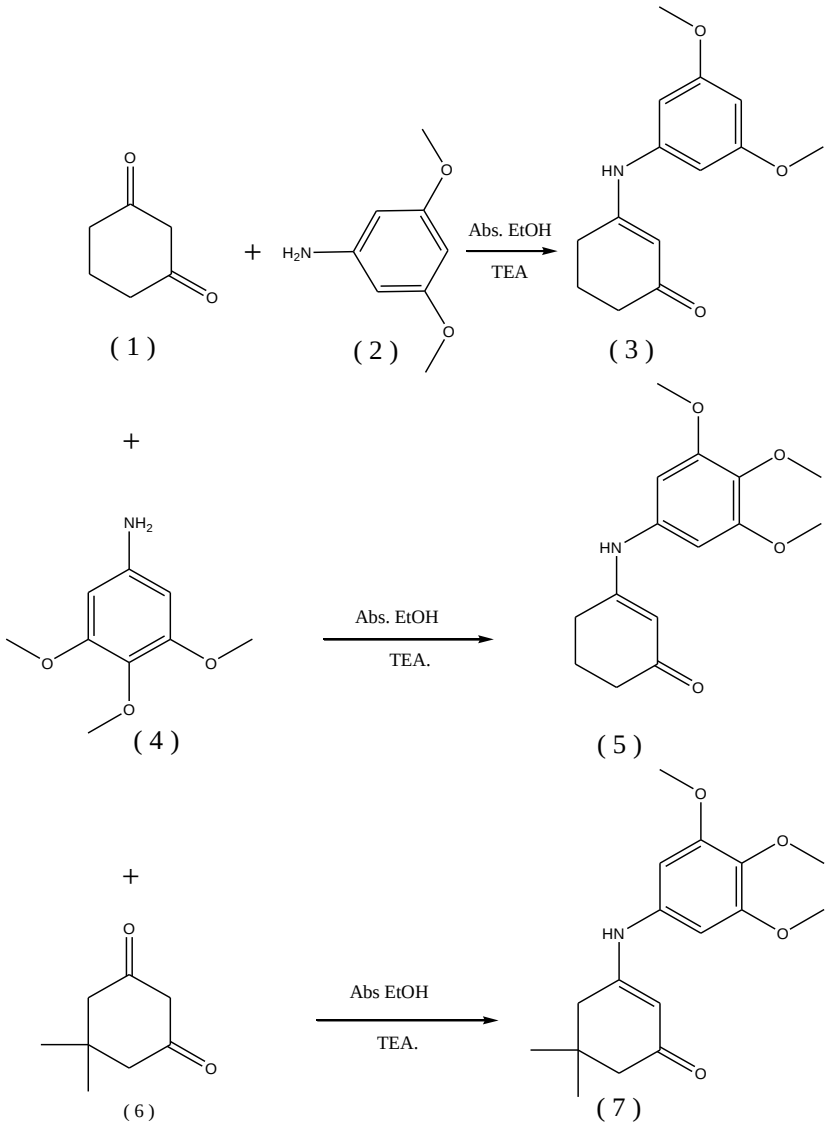
## Experimental

The starting materials cyclohexane-1,3- dione, 5,5- dimethylcyclohexane-1,3- dione, 3,4- dimethoxyaniline, and 3,4,5- trimethoxyaniline were purchased from Sigma-Aldrich. Melting points were determined on an electrothermal melting point apparatus (Stuart Scientific, Stone), and were uncorrected. Precoated Silica gel plates (Kiesel gel 0.25 mm, 60 G F 254, Merck) were used for thin layer chromatography (TLC). The developing solvent system was chloroform / methanol (10 : 3) and the spots were detected by ultraviolet light. Infrared (IR) spectra (KBr disc) were recorded on FT- IR spectrophotometer (Perkin Elmer) at the Research Center, College of Pharmacy, King Saud University, Saudi Arabia. $^{1}$H-NMR spectra were scanned in dimethylsulfoxide (DMSO-$d_6$) on a NMR spectrophotometer (Bruker AXS Inc.) operating at 500 MHz for $^{1}$H and 125.76 MHz for $^{13}$C at the aforementioned Research Center. Chemical shifts are expressed in d- values (ppm) relative to tetramethylsilane (TMS) as an internal standard. Exchangeable protons were confirmed by addition of a drop of $D_2O$. Elemental analyses were done on a model 2400 CHNSO analyzer (Perkin Elmer).

## Results

***Synthesis of 3-(3,5-dimethoxyphenylamino) cyclohex-2-enone*** (**3**).

A mixture of cyclohexane -1,3- dione **1** (1.22g, 0.01 mole) and 3,5- dimethoxyaniline **2** (1.53g, 0.01 mole) in absolute ethanol (30 mL) containing 3 drops of triethylamine was refluxed for 8h. The reaction mixture was cooled and the solid obtained was recrystallized from dioxane to give **3**. Yield % 94; m.p. 138-140 ºC; IR (KBr, $Cm^{-1}$): 3298 (NH), 3086 (CH arom.), 2972, 2876 (CH aliph.), 1652 (C=O). $^1$H-NMR spectrum in (DMSO-$d_6$): 1.39,1.87, .99 [m, 6H, $3CH_2$ cyclo.], 3.70 [s, 6H, 2 $OCH_3$], 5.7 [s, 1H, CH cyclo.], 5.9 – 6.3 [m, 3H, Ar-H], 9.7 [ s, 1H, NH, $D_2O$-exchangeable]. $^{13}$C-NMR spectrum in (DMSO-$d_6$): 19.6, 28.3, 38.8, 56.4 (2), 91.2, 92.6, 101.6, 145.9, 161.4, 163.7 (2), 200.6 (C=O). Anal. Calcd for $C_{14}H_{17}NO_3$ (247.29): C, 68.00; H, 6.93; N, 5.66. Found: C, 68.31; H, 6.64; N, 5.93**.**




( Scheme 1 )

***Table 1.*** *NQO1 inducer activity of enaminones*

| Compounds | Induction Magnitude (Fold) |
|---|---|
| 3-(3,5-dimethoxyphenylamino)cyclohex-2-enone (**3**) | 1.95 (at 100 µM) |
| 3-(3,4,5-trimethoxyphenylamino)cyclohex-2-enone (**5**) | 1.45 (at 80 µM) |
| 3-(3,4,5-trimethoxyphenylamino)-5,5-dimethylcyclohex-2-enone (**7**) | 1.67 (at 75 µM) |

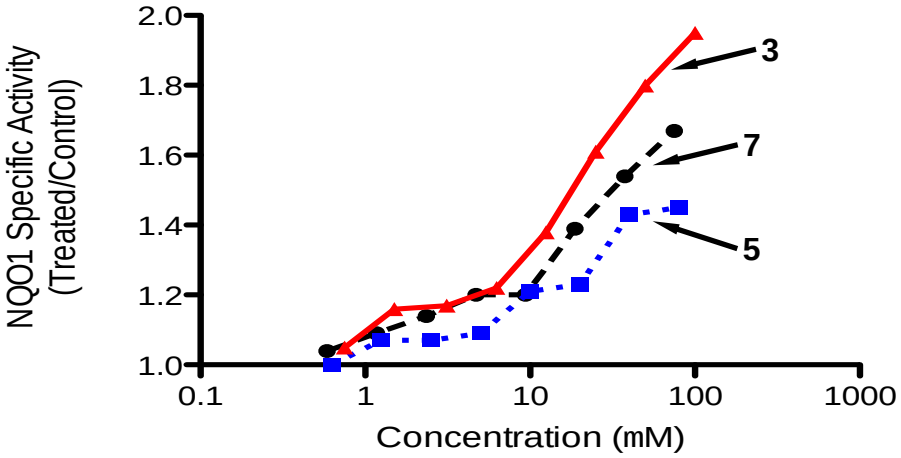



***Figure 1.*** *Dose response of NQO1 inducer activity of enaminones*

Hepa1c1c7 cells ($10^4$ per well) were grown in 96-well plates for 24 h. After that, the cell culture medium was replaced with fresh medium containing serial dilutions of enaminones. Cells were grown for a further 48h, and lysed with digitonin. The specific activity of NQO1 was determined in cell lysates using menadione as a substrate. Mean values for 8 replicate wells are shown for each data point. The standard deviation in each case was <5% of the value.

***Synthesis of 3-(3,4,5-trimethoxyphenylamino) cyclohex-2-enone*** ( **5** ).

A mixture of cyclohexane-1,3-dione **1** (1.12g, 0.01 mole) and 3,4,5- trimethoxyaniline **4** (1.83g, 0.01 mole) in absolute ethanol ( 30 mL) containing 3 drops of triethylamine was refluxed for 6h. The reaction mixture was cooled and the solid obtained was recrystallized from ethanol to give **5**. Yield % 89; m.p. 205-207 ºC; IR (KBr, $Cm^{-1}$): 3270 (NH), 3100 (CH arom.), 2966, 2836 (CH aliph.), 1653 (C=O). $^1$H-NMR spectrum in (DMSO-$d_6$): 1.8- 2.4 [m, 6H, $3CH_2$ cyclo.], 3.70, 3.78 [2s, 9H, 3 $OCH_3$], 5.2 [s, 1H, CH cyclo.], 6.4 [s, 2H, Ar-H], 8.7 [s, 1H, NH, $D_2O$- exchangeable]. $^{13}$C-NMR spectrum of **5** in (DMSO-$d_6$): 21.5, 28.4, 36.3, 55.8 (2), 60.0, 98.0 (2), 101.1, 134.4, 135.6, 153.0 (2), 162.2, 195.7 (C=O). Anal. Calcd for $C_{15}H_{19}NO_4$ (277.32): C, 64.97; H, 6.91; N, 5.05. Found: C, 64.69; H, 6.59; N, 5.31, melting point, IR, $^1$H-NMR, $^{13}$C-NMR and elemental analysis as reported by us[7].

***Biological evaluation***

The NQO1 inducer activity was determined using a quantitative microtiter plate assay [28]. Hepa1c1c7 cells were grown in αMEM supplemented with 10% (v/v) fetal bovine serum that had been heat- and charcoal-inactivated. Cells were routinely maintained in a humidified atmosphere at 37 °C, 5% $CO_2$. For each experiment, cells ($10^4$ per well) were plated in 96-well plates. After 24 h, the cell culture medium was replaced with fresh medium containing enaminones, and the cells were grown for a further 48 h. Eight replicates of 8 serial dilutions of each compound were used. Compounds were prepared as stock solutions in DMSO, and then diluted in the cell culture medium 1:1000. The final concentration of DMSO in the medium was maintained at 0.1% (v/v). At the end of the 48 h exposure period, cells were lysed for 30 min at 25 °C in digitonin (0.8 g/L, pH 7.8). The specific activity of NQO1 was evaluated in cell lysates using menadione as a substrate. Protein concentrations were determined in each well by the BCA protein assay (Thermo Scientific). Sulforaphane, a classical NQO1 inducer, served as a positive control.

## Discussion

The aim of the present work was the design, synthesis and structure elucidation of some enaminone derivatives carrying a biologically active 3,5-dimethoxyphenyl moiety 3 and 3,4,5-trimethoxyphenyl moieties 5 and 7 with expected anticancer activity (Scheme 1). 3-(3,5-Dimethoxyphenyl-amino)cyclohex-2-enone 3 was obtained in good yield via reaction of cyclohexane-1,3-dione 1 with 3,5- dimethoxyaniline 2 in refluxing ethanol containing a few drops of triethylamine as a catalyst (Scheme 1). The structure of compound 3 was supported by elemental analysis, IR, $^1$H-NMR, $^{13}$C-NMR spectra and x-ray data. IR spectrum of 3 revealed the presence of characteristic bands for NH at 3310 Cm-1, (CH arom.) at 3096 Cm-1, (CH aliph.) at 2977, 2866 Cm-1 and (C=O ) at 1647 Cm-1. Also, $^1$H-NMR spectrum in (DMSO-$d_6$) indicated the presence of a signals at 3.71 ppm which could be assigned to two methoxyl groups, 5.7 ppm due to CH cyclo., and 9.7 ppm for NH of enaminone 3. $^{13}$C-NMR spectrum of 3 in (DMSO-$d_6$) showed signals at 19.6, 28.3, 38.8, 56.4 (2), 91.2, 92.6 (2), 101.6, 145.9, 161.4, 163.7 (2), 200.6 (C=O).

In addition, interaction of 1 with 3,4,5-trimethoxyaniline 4 in refluxing ethanol afforded the corresponding 3-

(3,4,5-trimethoxyphenylamino)-cyclohex-2-enone 5 in good yield. The structure of 5 was confirmed from its microanalysis, IR, $^{1}$H-NMR, $^{13}$C-NMR and x-ray data. Thus, IR spectrum of 5 exhibited the presence of characteristic bands for NH, CH aromatic, CH aliphatic, and (C=O). $^{1}$H-NMR spectrum of 5 revealed signals at 3.70, 3.78 ppm corresponding to three methoxy groups, 5.2 ppm due to CH cyclo and 8.7 ppm for NH group. $^{13}$C-NMR spectrum of 5 in (DMSO-$d_6$) showed a signal at 195.7 (C=O). On the other hand, condensation of 5,5-dimethyl-cyclohexane-1,3-dione 6 with 3,4,5- trimethoxyaniline 4 yielded the corresponding 3-( 3,4,5- trimethoxyphenylamino)-5,5-dimethylcyclohex-2-enone 7 in good yield [23]. The structure of compound 7 was confirmed on the basis of elemental analysis, IR, $^{1}$H-NMR, $^{13}$C-NMR, and x-ray analysis. The IR spectrum of 7 showed bands for (NH), (CH aromatic), (CH aliphatic), and (C=O). Also, the $^{1}$H-NMR spectrum in (DMSO-$d_6$) indicated the presence of a singlet at 8.7 ppm which could be assigned to NH of enaminone 3.

Based on the electrophilicity of the cyclohexenone functionality, we tested the possibility that enaminones may be able to induce the cytoprotective enzyme NAD(P)H:quinone oxidoreductase 1 (NQO1). Indeed, the cyclopentenone prostaglandins are well-known endogenous activators of nuclear factor-erythroid 2 p45-related factor 2 (Nrf2), the main transcription factor which regulates the basal and inducible expression of NQO1 [29-32]. Using a quantitative bioassay in murine Hepa1c1c7 cells [28,33], we found that all three enaminones are weak inducers of NQO1 (Table 1 and Figure 1). Exposure of the cells to the enaminones for 48 h led to a dose-dependent upregulation of the specific activity of NQO1. The magnitude of induction among the three compounds was similar, with compound 3 being the most potent, and compound 5 being the least potent inducer. No cytotoxicity was observed at any of the tested concentrations of the enaminones.

The 3,4,5-trimethoxyphenyl moiety is an important structural feature for binding to tubulin and inhibition of microtubule polymerization, and is also present in the structure of colchicine [34]. In contrast, the similarity in inducer potency among the three enaminones indicates that the 3,4,5-trimethoxy substitution does not play a significant role for NQO1 induction as compound 3 lacks one of the methoxyl groups. This result points out to the importance of the cyclohexenone functionality, a feature shared by all three compounds. The electrophilic enone functionality was early recognized as a critical feature within a structurally diverse array of inducers of NQO1, due to the ability of enones to form Michael adducts with sulfhydryl groups [35]. By analogy with other NQO1 inducers bearing enone groups, such as phenylpropenoids, chalcones, curcuminoids and coumarins [36-38], we propose that the enaminones activate transcription factor Nrf2 by reacting with cysteine sensors of its major negative regulator, the ubiquitin ligase substrate adaptor Kelch-like ECH-associated protein 1 (Keap1). Under homeostatic conditions, Keap1 continuously targets Nrf2 for ubiquitination and proteasomal degradation [39-42]. Cysteine modification of Keap1 leads to a loss of its repressor function, ultimately resulting in Nrf2 stabilization, nuclear translocation, and activation of its target genes, such as NQO1.

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