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A Practical Synthesis and X-ray Crystallographic Analysis of Dithymoquinone, a Photodimer of Thymoquinone

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Abstract

An updated and practical approach to the synthesis of dithymoquinone via one-step photoirradiation of thymoquinone (2-methyl-5-isopropyl-1,4-benzoquinone) is described. Synthesis resulted in a 55% yield of one structural isomer (*trans-anti* derivative), as confirmed by HPLC, NMR spectroscopy and first ever single-crystal X-ray diffraction analyses.

Keywords

dithymoquinone; photodimerization; thymoquinone; X-ray structure

INTRODUCTION

Nigella sativa L. (Ranunculaceae), generally known as black seed or black cumin, has been used as a spice and natural remedy in folk medicine for over a century [1]. Pharmacological properties of black seed that have been reported include: anti-inflammatory, antimicrobial, analgesic, antihistaminic and antitumor activity [1]. In addition, black seed has been shown to protect against hepato-, nephro- and cardiotoxicity [2, 3]. Most of these activities have been attributed to benzoquinone compounds present within the seed [4], namely thymoquinone (**1**), thymohydroquinone (**2**) and dithymoquinone (**3**) (Fig. 1). Although their precise mechanism(s) of action remains unknown, some have postulated that these quinones

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exhibit their effects via free radical scavenging [5–7]. As the roles of these quinones interact in key biological and chemical processes continue to be studied experimentally, the demand for authentic standards of these compounds has increased. Hence, there is a pressing need for practical approaches to the synthetic preparation of these compounds.

Thymoquinone (2-isopropyl-5-methylbenzo-1,4-quinone; **1**) is a bright yellow crystalline compound that was first synthesized via oxidation of thymol (2-isopropyl-5-methylphenol) with hydrogen peroxide [8]. Compound **1** was originally isolated from the essential oil of black cumin seeds using silica gel chromatography by El-Dakhkhany in 1963 [9]. Highly pure (>99%) and relatively cost-efficient preparations of **1** can be purchased through commercial sources (e.g. Sigma-Aldrich Co.), but **2** and **3** are not as readily available.

Thymohydroquinone (2-methyl-5-propan-2-ylbenzene-1,4-diol; **2**), also referred to as dihydrothymoquinone or hydrothymoquinone, is the phenolic hydroquinone derivative of **1**. Earlier synthetic methods of **2** entailed converting **1** to its bisulphite derivative with sodium bisulphite, acidifying with dilute hydrochloric acid and crystallization from chloroform as colorless needle-like crystals [9]. The synthetic procedure of **2** was recently improved through one-step reduction of **1** by acetic acid in the presence of zinc catalyst and purification via sublimation [10]. This method has been reproduced in our laboratory with little difficulty (details not reported), so significant improvements or modifications to this procedure, in our opinion, are not necessary at this time. However, the practical synthetic preparation of **3** has not been described.

The first significant reports on the synthesis of dithymoquinone ((4b,8b-dimethyl-3,7-di(propan-2-yl)-4a,8a-dihydrobiphenylene-1,4,5,8-tetrone; **3**), a photodimer derivative of **1**, were published in the late 1800's [11, 12], and expanded upon by Smith and Tess in 1944 [13]. In the latter report a highly concentrated ethereal solution of **1** was evaporated to a thin, polycrystalline film and exposed to indirect daylight for seven days. After photo conversion, compound **3** was purified by crystallization in ethanol to pale yellow needle-like crystals [13]. Since then only slight modifications to these procedures have been published. In addition, these reports contain only brief and very limited descriptions of the synthesis [6, 7, 9, 14–17]. As our laboratory is interested in measuring the concentrations of **1**, **2** and **3** in commercial black seed oil preparations and their pharmacological activities *in vitro* and *in vivo* studies, we needed to be able to prepare multi-gram quantities of purified compound **3**. This was the impetus for our attempts to develop a concise and simple synthesis of **3**. Herein we describe a simple, practical synthesis of **3** which expands upon previous methods. Furthermore we report a detailed NMR spectroscopic and first ever, to our knowledge, single-crystal X-ray diffraction analysis of **3**.

RESULTS AND DISCUSSION

Photodimerization of quinones, such as naphthoquinones [18] and benzoquinones [19], has been known for some time. The photodimerization of thymoquinone (**1**) to dithymoquinone (**3**) was first described over 150 years ago [11, 12], yet significant details on its synthesis were last reported by Smith and Tess in 1944 [13]. With a growing body of scientific knowledge suggesting that **3** (as well as **1** and **2**) contribute to black seed's multi-modal

biological activities, there is a greater demand for these compounds for experimental use. Compound **1** is available commercially and the synthesis of **2** is well described [10]. Hence, our first goal was to synthesize **3** in an efficient, practical manner that improves upon previous syntheses; and secondly, use spectroscopy and X-ray diffraction to fully characterize the product.

Our synthesis commenced with dissolving **1** in a minimal volume of ethyl acetate. Two 32-watt linear fluorescent tube bulbs housed in a fume hood (Thermo Hamilton Safe Aire II) served as the light source, which is a practical approach for irradiation of compound **1**. Irradiation sources reported elsewhere include: diffused light [9], indirect sunlight [13, 14], daylight [7], 400 watt mercury vapor lamp [15] and a xenon lamp [16]. The solid state photodimerization reaction of **1** to **3** was monitored by reversed phase HPLC with ultraviolet detection. At select time points, small scrapings of crude compound from the sides of reaction vessel were diluted in methanol and injected into the HPLC system. The reaction was found virtually complete (>99.8%, based on disappearance of starting material) after 48 hours and only a single UV absorbing peak was observed by HPLC. To find a suitable crystallization solvent we conducted simple solubility experiments with a battery of organic solvents: methanol, ethanol, 2-propanol, *tert*-butanol, benzyl alcohol, acetonitrile, 1-chlorobutane, dichloromethane, dimethylformamide and cyclohexane. Among these solvents, 2-propanol showed the best crystallization behavior. We also found that a two solvent system consisting of dichloromethane:hexane, employed within a simplified vapor diffusion apparatus, produces hexagonal crystals of **3**. Practically, 2-propanol was used in our synthesis to crystallize **3** to fine, pale yellow crystals, and upon further washing, filtration and lyophilization resulted in a yield of 55%.

Solid state photodimerization of **1** to **3** has been shown previously to proceed via a [2+2] cycloaddition reaction [16]. This coupling reaction is putatively stereospecific, resulting in the anti head-tail (H-T) configuration of the cyclobutyl ring methyl groups [16]. However due to the symmetry of the quinoid rings, the stereospecificity of the molecule was only confirmed on the epoxy derivative of **3** [16]. Since photoirradiation products of substituted benzoquinones, such as the *cis-syn*, *cis-anti*, *trans-syn* and *trans-anti* dimers of 2,4-methyl naphthoquinone (menadione) [20] are possible, we sought to determine the stereochemistry of **3**. Our first attempt included 1-D and 2-D NMR spectroscopy, which included ¹H, ¹³C, COSY and HETCOR analyses (Fig. 2 and Fig. 3). Our NMR data are consistent with the formation of a single isomer of **3** from the photodimerization reaction, as we observed only one set of peaks in both ¹H and ¹³C NMR spectra. Furthermore, the olefinic methyl (δ 2.03 ppm, s) and proton of the same double bond (δ 6.18 ppm, s) of **1** (spectrum not shown) disappear upon irradiation and are accompanied by the appearance in the aliphatic region of the spectrum two new singlets (1.22 and 3.31). This result identifies the double bond involved in the dimerization. However, 1D and 2D Nuclear Overhauser Enhancement (NOE) experiments did not permit the assignment of the stereochemistry of the dimer about the cyclobutane ring (e.g. relative orientation of the cyclobutyl methyl groups).

X-ray diffraction data was collected on **3** to determine the relative orientation of the cyclobutane ring substituents. The crystal structure is shown in Fig. 4. The crystal structure of **3** belongs to the triclinic, space group P-1, and shows similarity to the previously

determined crystal structure of **1** [21]. The crystal structure confirms that the methyl groups on carbons C7 and C7' are oriented about the cyclobutane ring in the *trans-anti* configuration, and also supports earlier findings on the dithymoquinone-epoxide derivative [16].

CONCLUSION

In summary, an updated and efficient approach for the one-step synthesis of dithymoquinone (**3**), a bioactive component of black seed oil, has been achieved. NMR spectroscopy and X-ray crystallography confirmed that photodimerization resulted in one structural isomer of **3**, the *trans-anti* compound. This will facilitate others in the synthesis of **3** and similar dimers, as well as studies regarding the bioactivity of **3**.

EXPERIMENTAL

All chemicals and solvents were of ACS grade or better as available from commercial suppliers. Melting point data was measured on a Mettler Toledo FP62 automated melting point instrument. Infrared spectra were measured (solid-state) on a Bruker Alpha-P FT-IR instrument with a diamond ATR unit. HPLC was performed on an Agilent 1200 system coupled to a photodiode array detector (DAD SL). NMR spectra were recorded on a Bruker Advance 600 MHz spectrometer at the Translational Chemistry Core Facility (Department of Experimental Therapeutics). Mass spectra were obtained on an Agilent 6410 QQQ mass spectrometer. A Labconco FreeZone 4.5 L bench top freeze dry system was used for lyophilization.

General Procedure for the Synthesis of Dithymoquinone (**3**)

Compound **1** (0.50 g, 3.1 mmol) was dissolved in ethyl acetate (5.0 mL) in a 600 mL glass Pyrex beaker. The bright yellow solution was gently rotated along the inner surfaces of the beaker until complete evaporation to a thin, crystalline layer. The resulting thin layer (solid state) of **1** was exposed to fluorescent light (two Philips 700 series 32 watt bulbs) in a fume hood at room temperature. The photodimerization reaction of **1** to **3** was monitored by reversed phase HPLC. Separation was performed on a Zorbax SB-C18 analytical column (2.1x100 mm; 3.5 μ m) with a mobile phase (0.3 mL/min) consisting of 60:40 (methanol: 0.1% formic acid in water). Retention times (t_r) of **1** and **3** were 3.1 min and 3.9 min, respectively. The reaction was found to be >99% complete after 48 hours. Crude **3** was dissolved in a minimal volume of ethyl acetate, transferred to a smaller Erlenmeyer flask, and then evaporated to dryness over gentle heat and a stream of N₂. Crystallization of **3** was performed using 2-propanol to give fine, pale yellow needle-like crystals. Crystals were collected by centrifugal filtration (Centriplus YM-100 centrifugal filter devices spun at 4500 g for 10 minutes), washed with a small volume of ultra-pure water and cold 2-propanol, re-centrifuged and lyophilized overnight to dryness: **3** (275 mg, 55% yield, m.p. 200.7°C). UV_{max} 242 nm and UV_{min} 382 nm. IR (solid state): 3060 (vinylic C=C-H stretch), 2969–2872 (C-H stretch of aliphatic groups), 1668–1614 (C=O and C=C stretch), 1461, 1387, 1215, 1104, 1012, 920, 892 cm⁻¹. ¹H-NMR (600 MHz, CDCl₃): δ 6.71 (s, 2H), 3.31 (s, 2H), 3.14–3.07 (septet, J = 6.6, 2H), 1.22 (s, 6H), 1.16–1.13 (2d, J = 7.2, 6.6, 12H) ppm. ¹³C-NMR (150 MHz, CDCl₃): 198.8, 194.1, 161.5, 134.9, 54.8, 47.3, 27.2, 21.6, 21.0, 20.2 ppm.

Proton and carbon assignments were assisted by a ^1H , ^{13}C , COSY and HETCOR analyses (spectra not shown) of commercial **1**. ESIMS: 351.1 $[\text{M}+\text{Na}]^+$

X-ray Crystallography of **3**

Crystal Data for **3**: $\text{M} = 328.39$, $\text{C}_{20}\text{H}_{24}\text{O}_4$, triclinic, space group P-1, $a = 5.7756(14) \text{ \AA}$, $b = 9.215(2) \text{ \AA}$, $c = 9.399(2) \text{ \AA}$, $\alpha = 67.683(4)^\circ$, $\beta = 72.403(5)^\circ$, $\gamma = 72.605(4)^\circ$, $V = 431.39(17) \text{ \AA}^3$, $Z = 1$, $D_{\text{calc}} = 1.264 \text{ Mg/m}^3$. Crystals grew as pale yellow needles by slow evaporation from 2-propanol. The data crystal was cut from a larger crystal and had approximate dimensions: $0.47 \times 0.05 \times 0.04 \text{ mm}$. Diffraction data were collected on a Rigaku AFC12 diffractometer with a Saturn 724+ CCD using a graphite monochromator with $\text{MoK}\alpha$ radiation ($\lambda = 0.71075 \text{ \AA}$). A total of 1376 frames of data were collected using ω -scans with a scan range of 0.5° and a counting time of 29 seconds per frame. The data was collected at 100 K using a Rigaku XStream low temperature device and data reduction was performed using the Rigaku Americas Corporation's Crystal Clear version 1.40 software (The Woodlands, TX, USA). The structure was solved by direct methods using SIR97 [22] and refined by full-matrix least-squares on F^2 with anisotropic displacement parameters for the non-H atoms using SHELXL-97 [23]. Structure analysis was aided by use of the programs PLATON98 [24] and WinGX [25]. Most hydrogen atoms were calculated in ideal positions with isotropic displacement parameters set to $1.2 \times \text{Ueq}$ of the attached atom ($1.5 \times \text{Ueq}$ for methyl hydrogen atoms). The hydrogen atoms on C6 were observed in a 2F_max map and refined with isotropic displacement parameters. The function, $\sum w(|F_\text{o}|^2 - |F_\text{c}|^2)^2$, was minimized, where $w = 1/[(\sigma(F_\text{o}))^2 + (0.0598 \times P)^2 + (0.1148 \times P)]$ and $P = (|F_\text{o}|^2 + 2|F_\text{c}|^2)/3$. $R_\text{w}(F^2)$ refined to 0.125, with $R(F)$ equal to 0.0450 and a goodness of fit, $S = 1.11$. The data was checked for secondary extinction effects but no correction was necessary. Neutral atom scattering factors and values used to calculate the linear absorption coefficient are from the International Tables for X-ray Crystallography, Volume C [26]. X-ray crystallography was done at the X-ray Diffraction Laboratory (Department of Chemistry and Biochemistry, The University of Texas at Austin). All figures were generated using SHELXTL/PC, version 5.03 (Siemens Analytical X-ray Instruments, Madison, WI, USA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. (1).
The molecular structures of thymoquinone (1), thymohydroquinone (2) and dithymoquinone (3)

Fig. (2).

^1H NMR spectrum of pure **3**. Proton assignments, further confirmed by two dimensional COSY analysis (inset), are listed adjacent to each NMR peak, as well as within the chemical structure (inset). Chemical shifts are in good agreement to a previous analysis [7].

Fig. (3).
 ^{13}C NMR spectrum of purified **3**. Carbon (bearing protons) assignments confirmed by two dimensional HETCOR analysis (top-left inset) are shown adjacent to each peak. The chemical structure of **3** with carbon labeling scheme is also included (top-right inset).

Fig. (4).
X-Ray structure of **3**. The molecule sits around a crystallographic inversion center at $\frac{1}{2}, 0, 0$. Atoms with labels appended with a ' are related by $1-x, -y, -z$. Displacement ellipsoids are scaled to the 50% probability level.