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Isolation, characterization, and synthesis of some process-origin impurities of Atovaquone, a renowned anti-malarial drug

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Abstract

To improve the yield of Atovaquone, we explored the chemical consequences occurring during its step-wise synthesis (process chemistry). Hence, the entire focus of this initiative was aimed towards the isolation of a few major process origin byproducts that were formed during the two-step reaction process. The synthesis of Atovaquone was done by the use of commercially viable key starting material *trans*-4-(4-chlorophenyl) cyclohexanecarboxylic acid for the decarboxylative coupling reaction with another key reactant 2,3-dichloro-1,4-naphthoquinone to obtain the intermediate. The isolated intermediate was hydrolyzed to obtain Atovaquone. From the mother liquors (MLRs) of each stage, we were able to isolate a few major process origin impurities and characterize them with the assistance of spectral data. Additionally, we had

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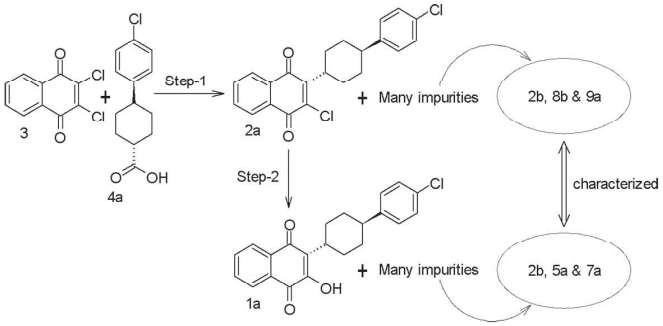
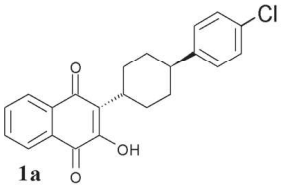
even synthesized some of the impurities and compared them with the ones that are isolated from the MLRs. In this work, we report the experimental aspects and results related to six process-origin impurities of Atovaquone. Too many impurities/byproducts are formed during the process, which substantiates for the moderate yield of the disclosed synthetic route to isolate Atovaquone. Isolation and characterization of all impurities formed will be an uphill task. Still, it will undoubtedly contribute to ascertaining the mass balance of the synthetic route under focus to get Atovaquone.

Keywords: Atovaquone, 2,3-Dichloro-1,4-naphthoquinone, Impurities, Isolation, Characterization, Synthesis.

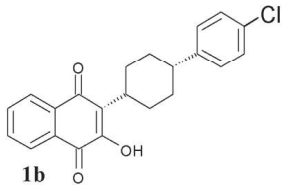
Graphical abstract



1 Introduction



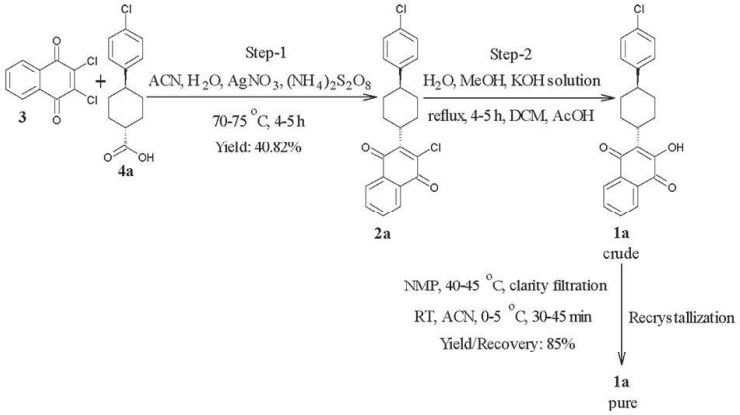
2-[*trans*-4-(4-chlorophenyl) cyclohexyl]-3-hydroxynaph thalene-1,4-dione



2-[*cis*-4-(4-chlorophenyl) cyclohexyl]-3-hydroxyna phthalene-1,4-dione

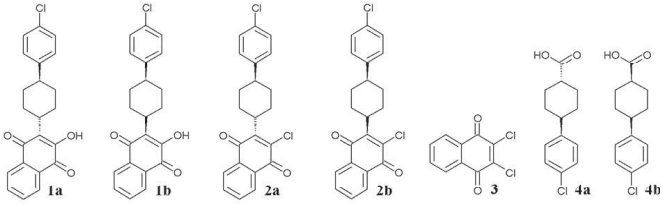
Atovaquone **1a** has the IUPAC name *trans*-2-[4-(4-chlorophenyl) cyclohexyl]-3-hydroxy-1,4-naphthoquinone with the trade name Mepron (CAS Registry No. 95233-18-4). Chemically it exists in two isomeric forms such as *trans*-form **1a** and the *cis*-form, 2-[*cis*-4-(4-chlorophenyl) cyclohexyl]-3-hydroxy-1,4-naphthoquinone **1b** (CAS Registry No. 137732-39-9) [1] (Malpezzi L., et al. 2010). **1a** is therapeutically active against un-complicated malaria [2] (Chiodini PL., et al. 1995), but **1b** is therapeutically inactive. This selectivity is due to significant differences among the isomers with respect to hydrogen bonding interaction between the amine-group of protein (host) and the carbonyl-group of **1a** or **1b** [3] (Basumallick S., et al. 2015). The spectroscopic analysis of **1a** binding with the parasite's mitochondrial cytochrome bc_1 complex (*cyt* bc_1) clearly demonstrates that **1a** attaches to *cyt* bc_1 in its ionized form. This sort of binding has been the actual reason for the antimalarial actions of **1a**. The side chains of more-conserved *cyt* b residues will form numerous non-polar interactions with napththoquinone part of **1a**. Likewise, less-conserved *cyt* b residues were in contact with the cyclohexyl–chlorophenyl extension of **1a**. More importantly, the polarized hydrogen bond to His181 of the Rieske protein in *cyt* bc_1 is in strong interaction with the ionized –OH group of **1a** [4] (Birth D., et al 2014). Usually, for better therapeutic efficacy **1a** is used along with another popular drug, Proguanil (CAS Registry No. 500-92-5) [5-7] (Looareesuwan S. et al. 1996; Malvy D., et al. 2002; Thybo S., et al. 2004). The synergistic antimalarial impact of **1a** and Proguanil had very high therapeutic efficacy in treating malaria since there was little evidence of resistance by the parasite if only **1a** were used for the treatment of malaria [8] (Srivastava IK., & Vaidya AB. 1999). A review article highlighted the drug development pathway, mechanism of action, resistance possibilities, metabolism, and the drug interactions of **1a,** which contribute towards its antimalarial actions [9] (Nixon GL., et al. 2013). It was even used to treat malaria in HIV-infected patients with much higher therapeutic success than the use of other drugs or drug combinations [10] (El-Sadr WM., et al. 1998). Some studies have even emphasized the role of **1a** as anti babesiosis, STAT3 inhibitor, anticancer agent, and also an antiviral agent to treat SARS-CoV-2 and other variants [11-13] (Krause PJ., et al. 2000; Xiang M., et al. 2016; Carter-Timofte ME., et al. 2021).

Our team successfully demonstrated the novel route for the synthesis of **1a** by the condensation of commercially viable starting material 2,3-dichloro-1,4-naphthoquinone **3** (Dichlone, CAS Registry No. 117-80-6) with *trans*-4-(4-chlorophenyl)cyclohexane carboxylic acid **4a** (CAS Registry No. 49708-81-8) through a two-step process (**Scheme 1**) in moderate yield with considerably high purity [14, 15] (Saralaya SS., et al. 2022).



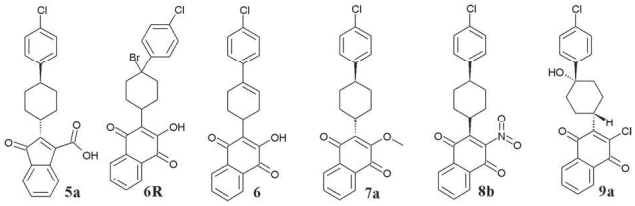
Scheme 1. Two-step process to synthesize **1a** from 2,3-dichloro-1,4-naphthoquinone (dichlone) **3** through decarboxylative coupling and hydrolysis.

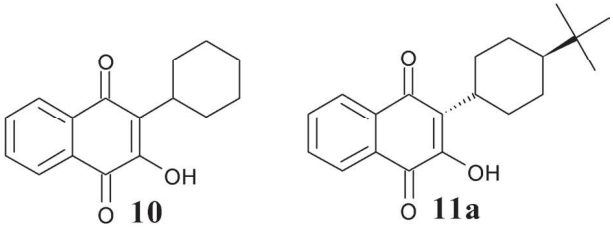
This optimized process (**Scheme 1**) involves the isolation of the intermediate 2-chloro-3-[*trans*-4-(4-chlorophenyl)cyclohexyl]-1,4-naphthoquinone **2a** (CAS Registry No. 153977-22-1) and its hydrolysis to get **1a**. The moderate process yield can be attributed to the formation of impurities in varied proportions. In the step-1 reaction, 2-chloro-3-[*cis*-4-(4-chlorophenyl)cyclohexyl]-1,4-naphthoquinone **2b** (CAS Registry No. 1071223-07-8) was found to be the major impurity, and in step-2 reaction, **1b** was identified as the major impurity formed in-situ during the process. To improve the yield, various attempts were reported by the different research groups through isomeric inter-conversions (via epimerization pathway) using different reagents and conditions. [16-20] (Shyam SV., et al. 2008; Antonio N., et al. 2010; Ashok K., et al. 2010; Fuqiang Z., & Michel B. 2011; Bhairab NR., et al. 2012).



Very limited prior arts are available towards the process-related/origin impurities of **1a**. Synthesis of impurity 2-[*trans*-4-(4-chlorophenyl) cyclohexyl]-1-oxo-1*H*-indene-3-carboxylic acid **5a** (CAS Registry No. 2517583-91-2) was reported by treating **1a** with 8% sodium hydroxide and recrystallizing from acetonitrile [21] (Chaniyara R., et al. 2013). Synthesis of impurity 2-[4-bromo-4-(4-chlorophenyl)cyclohexyl]-3-hydroxy-1,4-naphthoquinone **6R** was reported by the reaction of **2a** with *N*-bromosuccinamide (NBS) in carbon tetrachloride (CCl_4). Hydrolysis of **6R** by 10% potassium hydroxide solution, followed by acidification and purification by column chromatography, gave 2-[(1*RS*)-4-(4-chlorophenyl)cyclohex-3-en-1-yl]-3-hydroxy-1,4-naphthoquinone **6** (CAS Registry No. 1809464-27-4) **6** [22] (Deo K., et al. 2013). Synthesis, characterization, and pharmaceutical applications of 2-[*trans*-4-(4-chlorophenyl) cyclohexyl]-3-methoxy-1,4-naphthoquinone **7a** was reported along with some other naphthoquinone derivatives. It was prepared by treating **2**(**a**, **b**) with sodium in methanol and recrystallizing it from ethanol [23-25] (Victoria SA., et al. 1993; 1994; 1997). Its synthesis was also reported by treating **2a** with sodium in methanol and recrystallizing from acetonitrile [26] (Ravi C., et al. 2013). Synthesis of **7a** was disclosed by the reaction of **1a** with diazomethane in diethyl ether for a longer duration and purification by column chromatography [27] (Danoun S., et al. 1999). Our collaborative work discloses the studies of a few derivatives of **1a** with regard to crystal structure and binding capabilities with cyt *bc*1. This study involves the use of compounds like **1a**, **1b**, **2a**, **2b,** and 2-[*cis*-4-(4-chlorophenyl)cyclohexyl]-3-nitro-1,4-naphthoquinone **8b**, along with 2-cyclohexyl-3-hydroxy-1,4-naphthoquinone **10** (Parvaquone, CAS Registry No. 4042-30-2) and 2-(*trans*-4-*tert*-butylcyclohexyl)-3-hydroxy-1,4-naphthoquinone **11a** (Buparvaquone, CAS Registry No. 88426-33-9) [28] (Nayak SK., et al. 2013).

In this segment, we achieved the isolation of the above few impurities and characterized them with the assistance of spectral output. Additionally, we were able to isolate a new impurity 2-chloro-3-[*trans*-4-(4-chlorophenyl)-4-hydroxy-cyclohexyl]-1,4-naphthoquinone **9a** and characterized it with the resultant spectral support.





In our continuous efforts to synthesize **1a** from **3** in good yield, we observed the formation of numerous process origin impurities or byproducts, and they are the limiting factors towards reduced yield. Approximately 18-20 impurities in step 1 and around 5-7 impurities in step 2 were noticed by the assistance of thin-layer chromatography (TLC). Isolation and the characterization of these impurities could provide more dimensions to the reaction mechanism by which they form during the process. Additionally, the formation of these impurities significantly contributed to the lower yield of **1a**. A few process modifications to reduce or prevent the formation of these impurities can make the synthesis of **1a** commercially viable for large-scale manufacturing in good yield and purity.

2. Materials and Methods

2.1 Materials

The key starting material used in the present work, **3**, was procured from DL Intrachem (India), and the stereo-specific raw materials, like **4a** and **4b**, were procured from Sigma-Aldrich (India). The

catalytic reagent silver nitrate was procured from Rankem Chemicals (India). A few other essential chemicals like ammonium persulfate, sodium methoxide, potassium hydroxide, acetic acid, and sodium bicarbonate, were procured from SD Fine Chemicals (India). Silica gel (60-120 & 200-400) mesh was procured from LobaChemie (India) for column chromatography. The regular and pencil column chromatography glass apparatus were procured from Vital Science Industry (India). The analytical grade TLC plates having silica gel ($^{60}F_{254}$) coated over aluminum foil were procured from Merck (India). All the required solvents were procured locally in commercial grades. The procured chemicals/reagents and solvents were used directly for the experiments without further purification.

2.2 Instruments

Melting points (mp) of all the isolated and synthesized impurities were recorded by the open capillary method and are uncorrected. IR spectra were recorded on Mettler-Toledo FT-IR (ATR, React-IR 702L). The UV spectrum was recorded on the Mettler-Toledo Spectrophotometer Easy VIS. Mass spectra were recorded on an Agilent Technology LC-Mass Spectrometer. ^{1}H NMR and ^{13}C NMR spectra were recorded (in DMSO-d_6/$CDCl_3$) on a Bruker Advance (300/400 MHz) spectrometer using trimethylsilane (TMS) as an internal standard. Coupling constants *J* are in Hz, and multiplicities are represented as a singlet (s), doublet (d), triplet (t), broad singlet (bs), and multiplet (m). Progress of the reaction, purity of the products, fractions, and the isolated impurities were checked by TLC, using pre-coated silica TLC plates ($^{60}F_{254}$). The spots on the TLC plates were viewed over a short-wavelength (254 nm) UV light cabinet (Coral Labtech Enterprises, India).

2.3 Methods

2.3.1 Procedure to isolate impurities from Step 1

Reaction in step 1 involves the condensation of **3** (30.00 g, 0.132 mol) with **4a** (31.54 g, 0.132 mol) to isolate **2a**. Isolated product weight: 20.78 g, yield: 40.82%, mp: 184-186°C (acetonitrile) [15] (Saralaya SS., et al. 2022). The filtrate obtained after isolation of **2a** was extracted with 100 mL of dichloromethane and then concentrated under reduced pressure to get 13.00 g of the dark syrupy residue. This residue was

subjected to column chromatography using 400.00 g of silica gel 60-120 mesh and a mixture of n-hexane and ethyl acetate in the ratio of 9:1 as an eluent. Based on the polarity factor, three different fractions with impurities are collected for further processing. The fractions composing non-polar impurities were concentrated under reduced pressure to get the residue, RS-1 (470 mg). Similarly, the fractions having semi-polar impurities gave RS-2 (287 mg), and the fractions with polar impurities gave RS-3 (4.50 g).

Around 250 mg of RS-1, RS-2, and RS-3 were subjected to separate column chromatographic purification using 50.00 g of silica gel (200-400 mesh) using n-hexane and ethyl acetate (by gradually raising the polarity) as eluent, to enrich major constituents to a purity of around 90% by TLC. Further, it was purified by repeating the chromatographic process using a pencil column. The pencil column was prepared by using 12.00 g of silica gel (200-400 mesh) and the mixture of n-hexane and ethyl acetate as eluent in the ratio 9.5:0.5. Each pure fraction was evaporated to dryness under reduced pressure at 35-40^{o}C to isolate the respective impurities (29 mg of **2b**, 22 mg of **9a** and 30 mg of **8b**). A TLC of isolated impurities, eluted by toluene and ethyl acetate (8.5:1.5), gave the Rf values as 0.8 for **2b**, 0.35 for **9a,** and 0.2 for **8b,** respectively.

2.3.2 Procedure to isolate impurities from Step 2

Reaction in step 2 involves the hydrolysis of **2a** (19.00 g, 0.05 mol), followed by acidification and recrystallization to isolate **1a**. Isolated product weight: 14.64 g, yield: 81.00%, mp: 218-220^{o}C (acetonitrile & NMP) [15] (Saralaya SS., et al. 2022). The filtrate obtained after crude isolation FL-1 was extracted into dichloromethane and washed with 40 mL of DM water. It was mixed with the filtrate obtained after the isolation of the product FL-2. This filtrate mixture (FL-1+FL-2) was concentrated under reduced pressure to get the sticky residue. A portion of residue (8.00 g) was subjected to column chromatography using 250.00 g of silica gel (200-400 mesh) and eluted by the mixture of ethyl acetate and methanol in the ratio 9:1. Two components were collected, and the fractions having non-polar impurities on solvent evaporation gave the residue, RS-4 (540 mg). Likewise, the fractions having polar impurities on evaporation of the solvent gave the residue RS-5 (2.70 g).

Around 500 mg of RS-1 and RS-2 were subjected to a distinct column chromatography process executed by the use of 50.00 g of silica gel 200-400 mesh and eluted by n-hexane, ethyl acetate, and methanol (by gradually raising the polarity) to enrich each one of them to a purity of around 90% by TLC. Furthermore, each of those was passed through a separate pencil-column chromatography process to eliminate the minor traces of closely associated impurities. The pencil-column chromatogram was prepared by using 10.00 g of silica gel 200-400 mesh and eluted by the mixture of toluene and ethyl acetate in the ratio of 9.5:0.5. Each pure fraction was evaporated to dryness under reduced pressure at 35-40°C to isolate the respective impurities (22 mg of **1b**, 17 mg of **7a** and 28 mg of **5a**). A co-spot TLC of isolated impurities, eluted by toluene and ethyl acetate in the ratio of 8:2, gave the Rf values as 0.7 for **1b**, 0.5 for **7a,** and 0.15 for **5a,** respectively.

2.4 Characterization of isolated impurities

2.4.1 2-Chloro-3-[cis-4-(4-chlorophenyl) cyclohexyl]-1,4-naphthoquinone 2b

Yellow solid, ^{1}H NMR (400 MHz, $CDCl_3$), δ, ppm (*J*, Hz): 8.02-8.12 (2H, m, *β*-naphthoquinyl), 7.70-7.75 (2H, m, *α*-naphthoquinyl), 7.39-7.41 (2H, d, phenyl, *J*=8.4), 7.33-7.35 (2H, d, phenyl, *J*=8.8), 3.37-3.42 (1H, m, cyclohexyl), 3.14 (1H, bs, cyclohexyl), 2.26-2.37 (4H, m, cyclohexyl), 1.92-1.98 (2H, m, cyclohexyl), 1.51-1.54 (2H, m, cyclohexyl). Melting point: 174-176°C (ethyl acetate), molecular formula: $C_{22}H_{18}Cl_2O_2$.

2.4.2 2-[cis-4-(4-Chlorophenyl) cyclohexyl]-3-nitro-1,4-naphthoquinone 8b

Bright yellow solid, ^{1}H NMR (300 MHz, DMSO-d_6), δ, ppm (*J*, Hz): 8.02-8.05 (2H, m, *β*-naphthoquinyl), 7.91-7.94 (2H, m, *α*-naphthoquinyl), 7.42 (4H, s, phenyl), 3.08 (1H, s, cyclohexyl), 2.70-2.73 (1H, m, cyclohexyl), 1.56-2.27 (8H, m, cyclohexyl). ^{1}H NMR (300 MHz, $CDCl_3$), 8.02-8.07 (2H, m, *β*-naphthoquinyl)), 7.73-7.76 (2H, m, *α*-naphthoquinyl)), 7.28 (4H, s, phenyl), 3.07 (1H, bs, cyclohexyl), 2.61-2.69 (1H, m, cyclohexyl), 1.18-2.21(8H, m, cyclohexyl), MS (*m/z*): 413.1 (ammonia adduct). Melting point: 175-177°C (ethyl acetate), molecular formula: $C_{22}H_{18}ClNO_4$.

2.4.3 2-Chloro-3-[trans-4-(4-chlorophenyl)-4-hydroxycyclohexyl]-1,4-naphthoquinone 9a

Pale yellow solid, IR (KBr, γ max, cm^{-1}): 3445.32 (hydroxy), 1661.77 (carbonyl), ^{1}H NMR (400 MHz, $CDCl_3$), δ, ppm (*J*, Hz): 8.10-8.15 (2H, m, *β*-naphthoquinyl), 7.72-7.89 (2H, m, *a*-naphthoquinyl), 7.49-7.51 (2H, d, phenyl, *J*=8.4), 7.32-7.34 (2H, d, phenyl, *J*=8.4), 3.34-3.42 (1H, m, cyclohexyl), 2.62-2.72 (2H, m, cyclohexyl), 1.65-1.98 (7H, m, cyclohexyl). DEPT-*135*, *90*, and *45* spectra had identified CH, CH_2, and CH_3 carbons. As expected, CH and CH_3 carbon signals were up (positive), and CH_2 was down (negative) in DEPT-*135*. Likewise, CH was up, CH_2 and CH_3 did not show any signals in DEPT-*90*, and CH, CH_2 and CH_3 were up in DEPT-*45*. From these leads, we could determine and allocate C atoms in ^{13}C NMR. ^{13}C NMR (400 MHz, $CDCl_3$), δ, ppm: 182.63 (C-15, naphthoquinyl), 177.81 (C-18, naphthoquinyl), 150.16 (C-19, naphthoquinyl), 147.46 (C-11, chlorophenyl), 143.34 (C-14, naphthoquinyl), 134.28 (C-21, naphthoquinyl), 133.82 (C-22, naphthoquinyl), 132.65 (C-16 naphthoquinyl), 132.26 (C-17, naphthoquinyl), 130.98 (C-8, chlorophenyl), 128.38 (C-9 & C-13, chlorophenyl), 127.12 (C-20, naphthoquinyl), 127.05 (C-23, naphthoquinyl), 126.05 (C-10 & C-12, chlorophenyl), 71.98 (C-1, cyclohexyl), 39.97 (C-4, cyclohexyl), 38.66 (C-2 & C-6, cyclohexyl), 24.34 (C-3 & C-5, cyclohexyl). The signal at 71.98 ppm in the ^{13}C NMR spectrum was assigned to the C atom of the cyclohexyl ring substituted to the 4-chlorophenyl ring. The signal at 39.97ppm was assigned to the C atom of the cyclohexyl ring substituted to naphthoquinyl ring. The ^{13}C-HSQC spectrum showed an interaction of C and H at 39.97ppm, whereas the interaction of C at 71.98ppm with H was absent, hence confirming the proposed structure. MS (*m/z*): 425.04 (sodium adduct). Melting point: 205-207°C (ethyl acetate), molecular formula: $C_{22}H_{18}Cl_2O_3$.

2.4.4 2-[cis-4-(4-Chlorophenyl) cyclohexyl]-3-hydroxy-1,4-nahthoquinone 1b

Yellow solid, ^{1}H NMR (400 MHz, DMSO-d_6), δ, ppm (*J*, Hz): 8.03-8.14 (2H, m, *β*-naphthoquinyl), 7.64-7.78 (2H, m, *a*-naphthoquinyl), 7.16-7.39 (4H, m, phenyl), 3.09-3.22 (1H, m, cyclohexyl), 2.38-2.63 (2H, m, cyclohexyl), 2.13-2.39 (2H, m, cyclohexyl), 1.48-1.97 (5H, m,

cyclohexyl). MS (m/z): 365.0. Melting point: 129-131°C (ethyl acetate & methanol), molecular formula: $C_{22}H_{19}ClO_3$.

2.4.5 2-[trans-4-(4-Chlorophenyl) cyclohexyl]-1-oxo-1H-indene-3-carboxylic acid 5a

Shiny yellow solid, IR (KBr, γ max, cm^{-1}): 3404.98 (-OH of carboxylic acid), 1711.84 (carbonyl of carboxylic acid), 1685.45 (carbonyl of indene). ^1H NMR (400 MHz, DMSO-d_6), δ, ppm (*J*, Hz): 13.92 (1H, bs, carboxylic acid), 7.43-7.52 (3H, m, aromatic), 7.27-7.36 (5H, m, aromatic), 3.05-3.13 (1H, m, cyclohexyl), 2.50-2.62 (1H, m, cyclohexyl), 1.52-2.01 (8H, m, cyclohexyl). ^{13}C NMR, (400 MHz, DMSO-d_6), δ, ppm: 197.09 (C-8, indene), 165.53 (C-23, carboxylic acid), 145.98 (C-7, indene), 144.27 (C-11, indene), 143.45 (C-9, indene), 142.57 (C-10, indene), 134.53 (C-19, chlorophenyl), 130.32 (C-13, indene), 129.13 (C-14, indene), 128.60 (C-15 & C-12, indene), 128.53 (C-20 & C-18, chlorophenyl), 128.08 (C-16, chlorophenyl), 122.79 (C-17, chlorophenyl), 122.21 (C-21, chlorophenyl), 42.33 (C-1, cyclohexyl), 34.85 (C-4, cyclohexyl), 33.45 (C-3 & C-5, cyclohexyl), 29.85 (C-2 & C-6, cyclohexyl). UV (λ max): 244 nm. MS (m/z): 365.04. Melting point: 289-291°C (ethyl acetate & methanol), molecular formula: $C_{22}H_{19}ClO_3$.

2.4.6 2-[trans-4-(4-Chlorophenyl) cyclohexyl]-3-methoxy-1,4-naphthoquinone 7a

Yellow solid, IR (KBr, γ max, cm^{-1}): 2408.42 (methoxy), 1681.68 (carbonyl). Purity (a% by HPLC): 93.36%. ^1H NMR (400 MHz, $CDCl_3$), δ, ppm (*J*, Hz): 8.03-8.08 (2H, m, *β*-naphthoquinyl), 7.68-7.73 (2H, m, *a*-naphthoquinyl), 7.26-7.28 (2H, d, phenyl, *J*=8.4), 7.17-7.19 (2H, d, phenyl, *J*=8.4), 4.11 (3H, s, methoxy), 3.15-3.22 (1H, m, cyclohexyl), 2.59-2.66 (1H, m, cyclohexyl), 2.10-2.17 (2H, m, cyclohexyl), 1.95-2.08 (2H, m, cyclohexyl), 1.72-1.76 (2H, m, cyclohexyl), 1.52-1.62 (2H, m, cyclohexyl). Melting point: 126-128°C (ethyl acetate & methanol), molecular formula: $C_{23}H_{21}ClO_3$.

2.5 Synthetic routes to prepare impurities

2.5.1 Synthesis of 2b and 1b

Condensation of **3** (5.00 g, 0.022 mol) with **4b** (5.25 g, 0.022 mol) was carried out in a 500 mL 3-necked round bottom flask using silver nitrate (3.73 g, 0.022 mol) and ammonium persulfate (15.06 g, 0.066

mol) in 50 mL of acetonitrile and 65 mL of DM water at 70-75°C for 5-6 h. It was cooled to ambient temperature after the completion of the reaction and filtered to isolate the crude product with inorganics. 50 mL of dichloromethane was added, stirred, and filtered to remove the inorganic salts. The organic layer was washed with 15 mL of 10% sodium bicarbonate solution, followed by 30 mL DM water wash, and the solvent was evaporated. The residue was triturated with 15 mL of acetonitrile, and the resulting solid was isolated by filtration to get **2b**. Isolated product weight: 3.01 g, yield: 35.4%, mp: 174-176°C (acetonitrile). Hydrolysis of **2b** (2.80 g, 0.0072 mol) in the presence of aqueous potassium hydroxide (2.75 g, 0.049 mol, dissolved in 28 mL of DM water) in 50 mL of methanol for 4-5 h under reflux. After the reaction completion, the reaction mass was cooled to ambient temperature and acidified with dilute hydrochloric acid till pH 2-3 to get a yellow precipitate. It was filtered and washed with DM water to isolate **1b**. Isolated product weight: 1.92 g, yield: 72.55%, mp: 130-133°C (ethyl acetate).

2.5.2 Synthesis of 5a

Hydrolysis of **2a** (3.00 g, 0.008 mol) was carried out using an aqueous potassium hydroxide solution (4.49 g, 0.08 mol in 15 mL of DM water) in 60 ml of 1,4-dioxan. It was refluxed at 95-100°C for 5 days, cooled to ambient temperature, and acidified by hydrochloric acid to pH 2-3 to get a yellow precipitate. It was cooled to 0-5°C and filtered to obtain crude **5a** (2.70 g). It was purified by column chromatography by using 150.00 g of silica gel (230-400 mesh) and n-heptane and ethyl acetate as eluent, in the ratio of 9:1. It was further purified by column chromatography using ethyl acetate and methanol in the ratio of 8.5:1.5. Solvent was evaporated to dryness to isolate **5a**. Obtained weight: 31.00 mg, yield: 1.0%., mp: 290-291°C (ethyl acetate & methanol).

2.5.3 Synthesis of 7a

In a 250 mL 3-necked round bottom flak, **2a** (2.00 g, 0.0052 mol) and sodium methoxide (1.40 g, 0.026 mol) were reacted in 20 mL of methanol under reflux for 4-5 h. The reaction mass was cooled to ambient temperature and extracted into 30 mL of dichloromethane. The dichloromethane layer was washed with 2% sodium hydroxide

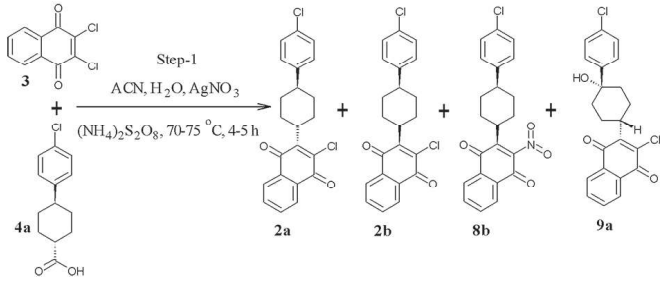
solution and later with DM water. The solvent was evaporated to dryness to isolate **7a**. Obtained weight: 1.19 g, yield: 60.19%, mp: 129-131°C (dichloromethane).

By TLC, the Rf values of the above compounds were compared with the ones that were isolated from column chromatography. For a few compounds, IR and ^{1}H NMR spectra were taken for the confirmation of molecular structure.

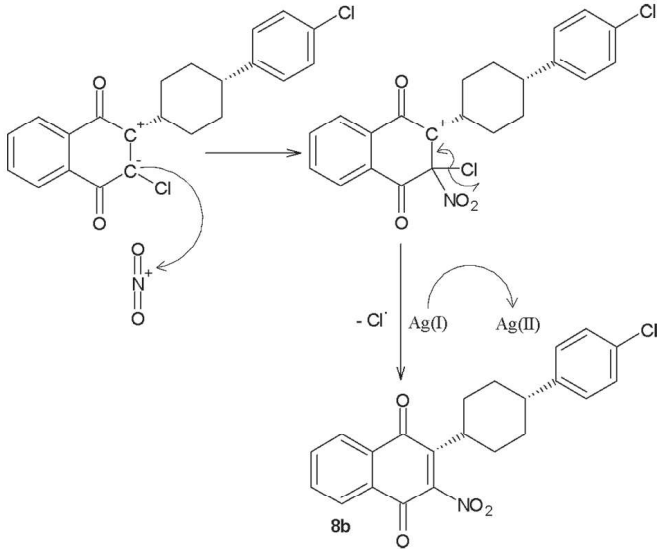
3 Results and Discussion

3.1 Chemistry behind the formation of 2b, 8b and 9a

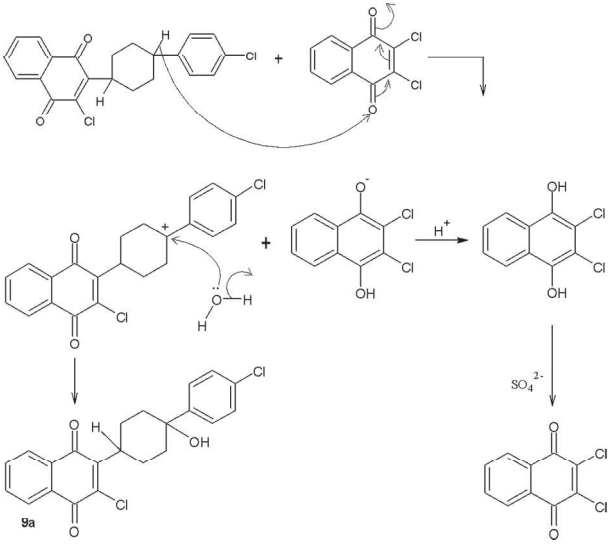
Interaction of the starting material, *trans*-4-(4-chlorophenyl)cyclohexyl carboxylic acid **4a** with silver nitrate in aqueous acetonitrile medium results in the formation of 4-(4-chlorophenyl) cyclohexyl free radical. This free radical undergoes a coupling reaction with 2,3-dichloro-1,4-naphthoquinone **3** in both *cis* and *trans* configurations to give **2a** and **2b,** respectively. The formation of **2b** has been less favored over its required stereoisomer **2a**. However, during the process, a substantial quantity of **2b** (30-35% by TLC) was formed. Thus, **2b** becomes a major impurity in step 1 (**Scheme 2**). Meanwhile, the cation (NO_2^+) reacts with **2b** to displace the chlorine atom in the presence of silver nitrate to form **8b** (**Scheme 3**) [29] (Olah GA., et al. 1982). Furthermore, the abstraction of hydrogen associated with cyclohexyl ring of **2a** by **3** and substitution of hydroxyl ion (OH^- was formed by the cleavage of water) to the cyclohexyl ring results in the formation of **9a** (**Scheme 4**) [30] (Chen L-Y., et al. 2010). Due to the formation of numerous impurities, the yield of **2a** had reached only to an extent of 38-43%. The drop-wise addition of ammonium persulfate to the reaction mass gave a poor yield of around 15-20%, but charging all the reagents together gave us a better yield of around 38-43%. Probably the free radicals generated during this mode of addition had been used up instantly for the reaction to form the required product **2a**, before they contributed to the formation of impurities through rapid radical disintegration.



Scheme 2. Decarboxylative coupling of **4a** with **3** to form **2a** and a few impurities (Step-1).



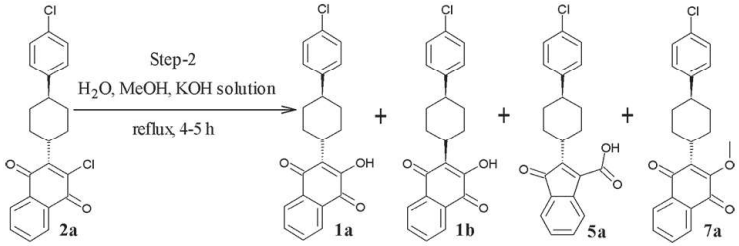
Scheme 3. A possible reaction mechanism for the formation of **8b**.



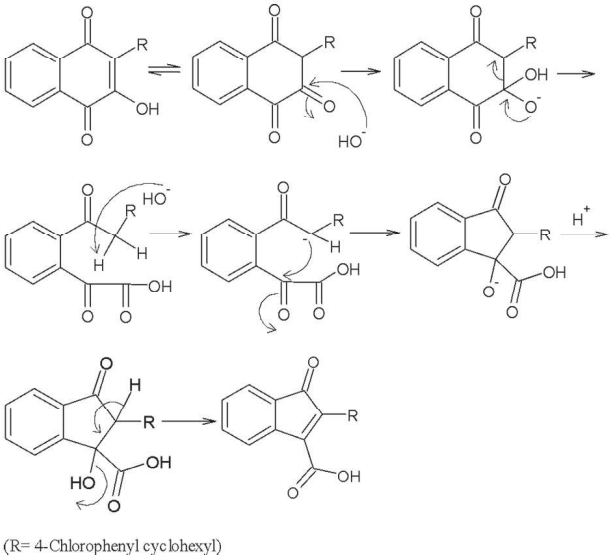
Scheme 4. A possible reaction mechanism for the formation of **9a**

3.2 Chemistry behind the formation of 1b, 5a and 7a

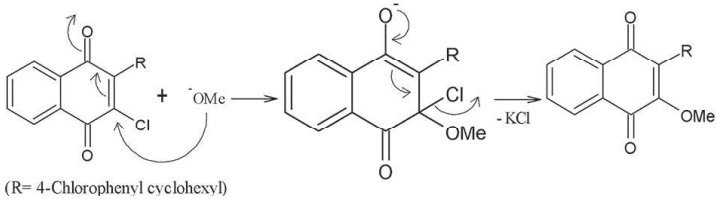
Step-2 reaction involves the hydrolysis of **2a** in the presence of potassium hydroxide solution in aqueous methanol (6.85 mol equiv) under reflux for 4-5 h (**Scheme 5**). Exposure of reactants to a strong alkaline medium at a relatively high temperature led to the formation of some impurities along with **1a**. A trace amount of **2b** existed in the input raw material, which would get hydrolyzed to form **1b** (around 0.5-1.0% by TLC). Alkali-mediated ring contraction of 1,4-naphthoquinone to indene had resulted in the formation of **5a** (1.0-1.5% by TLC) [31] (Cooke RG., & Somers TC. 1950). A few other research groups had reported the characteristic naphthoquinone ring contraction, and it was achieved under the impact of strong metal catalysts like palladium and cobalt [32, 33] (Wang L., et al. 2016; Eyong KO., et al. 2012). Use of methanol and alkali will favour the formation of **7a** to around 1-2% by TLC during the hydrolysis [34] (de Oliveira MF., et al. 2011). Possible or feasible reaction mechanisms were depicted for the formation of **5a** (**Scheme 6**) and **7a** (**Scheme 7**). These impurities were prepared (**Scheme 8**) by stimulating the favorable conditions for their formation, and they were compared with the previously isolated impurities.



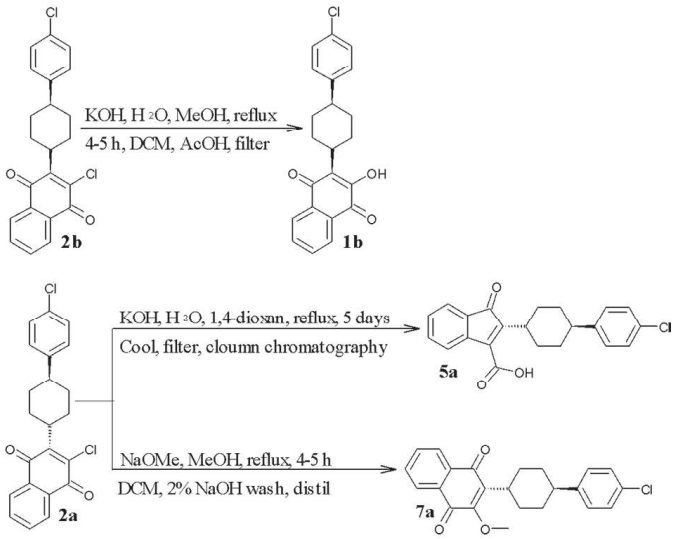
Scheme 5. Hydrolysis of **2a** to obtain **1a** and impurities like **1b**, **5a** & **7a**.



Scheme 6. A possible reaction mechanism for the formation of **5a**.



Scheme 7. A possible reaction mechanism for the formation **7a**



Scheme 8. Synthesis of **1b** from **2b**, **5a** and **7a** from **2a**.

Conclusions

In view of understanding the reason behind the moderate yield of **1a** during its synthesis starting from **3**, we undertook the task of isolating some of the impurities formed during the reactions (step 1 and step 2). Six of such impurities i.e. **2b**, **8b,** and **9a** from step-1 and **1b**, **5a,** and **7a** from step-2, were isolated and characterized by the spectral support. Furthermore, some of the impurities were synthesized, such as **1b**, **5a,** and **7a,** and they were compared with the isolated impurities to authenticate their structure. Some other impurities were also present in trace amounts and their structure elucidation could be ventured. Further studies to isolate all the process origin impurities and their characterization would be more challenging, but it would provide more insight into the chemistry around the synthesis of **1a**. These details would eventually assist in further optimizing the reaction conditions for the manufacturing of this crucial drug in a large scale with higher yield and better purity.

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Supplementary information (SI)

The spectral details that are supportive of structural elucidation of isolated impurities/byproducts are available in the SI file (**Figure S1** to **S30** and **Table S1** to **S6**, page no. 3 to 29).

Author contributions

Sanjay had collected the prior arts, performed experiments, analyzed the result outputs, and wrote the entire manuscript. *Shashikumar* helped perform experiments and perform prior art analyses. Akshaya assisted in performing experiments and isolating compounds through column chromatography. *Shashiprabha* assisted in writing the manuscript and spectral analysis (molecular structure interpretations). *Shridhara* helped design experiments and make manuscript corrections.

Conflict of Interest

The authors hereby declare no potential conflicts of interest with respect to the research, funding, authorship, and/or publication of this article

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