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SYNTHESIS AND HIV-1 REVERSE TRANSCRIPTASE INHIBITION ACTIVITY OF 1,4-NAPHTHOQUINONE DERIVATIVES

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*Some 1,4-napthoquinone derivatives were synthesized, and dimers of 5-hydroxy-7-methyl-1,4-naphthoquinone (**5**) were isolated from the roots of* Euclea natalensis*. Their structures were confirmed by spectroscopic (UV, IR, NMR, and MS) analysis. The HIV-1 reverse transcriptase inhibition activity of the compounds against recombinant HIV-1 enzyme was studied* in vitro *(non-radioactive HIV-RT colorimetric assay) using the Roche Diagnostic kit and compared with that of doxorubicin as standard drug. Some of the synthesized compounds exhibited exceptionally potent (91–100%) HIV-1 RT inhibition at 100 μg/mL concentration. Surprisingly the dimers showed very weak activity.*

Keywords: HIV-1 reverse transcriptase, anti-HIV activity, cytotoxic activity, 1,4-napthoquinone, 7-methyljuglone, diospyrin, isodiospyrin, dimer, *E. natalensis*.

Acquired immune deficiency syndrome (AIDS) is the most pandemic disease of the molecular biology era, which killed over 20 million people worldwide. The human immunodeficiency virus (HIV) is the AIDS causative agent, and its reverse transcriptase (HIV RT) is the main target of AIDS treatment. This enzyme has an important role in the virus replication. It permits the transcription of HIV-single-stranded RNA genome into a DNA double helix capable of integration into host cell chromosomes [1].

There is a decline in morbidity and mortality due to the combination of HIV reverse transcriptase and protease inhibitors in the highly active anti retroviral therapies (HAART) against AIDS-HIV infection. The lymphatic system and central nervous system act as reservoirs for the virus, hence viral replication still persists [2], and some antivirals, particularly the protease inhibitors (PIs), do not penetrate at an efficient inhibitory level [3].

In the past two decades, a worldwide search has been made for new chemotherapeutic agents targeting the HIV. The USA Food and Drugs Administration (FDA) has approved 19 new drugs including nucleoside analogue HIV reverse transcriptase [4], non-nucleoside RT inhibitors [5], protease inhibitors [6], and fusion inhibitors [7] for clinical use in the world. However, this existing drug therapy encounters problems, such as drugs having only limited or transient clinical benefit due to their side effects, the emergence of drug resistant viral strains, and toxicity effects due to long-term drug usage [8]. Also azidothymidine (AZT) does not destroy the HIV infection but only delays the progression of the disease and the replication of virus even at higher doses. During prolonged AZT treatment, HIV becomes resistant to AZT through mutation of the reverse transcriptase. Hence AZT is given in combination with some RT inhibitors and an antiretroviral from another group such as a protease inhibitor or a non-nucleoside RT inhibitor [9].

In addition, the emergence of drug-resistant virus strains due to high HIV mutation rates continues to restrain the long-term clinical efficacy of these molecules, whereas side effects limit the use of some of these antivirals (i.e., AZT), requiring new options. Hence, though there are developments in treating the progression of AIDs in HIV-positive individuals, it still remains a major health problem worldwide.

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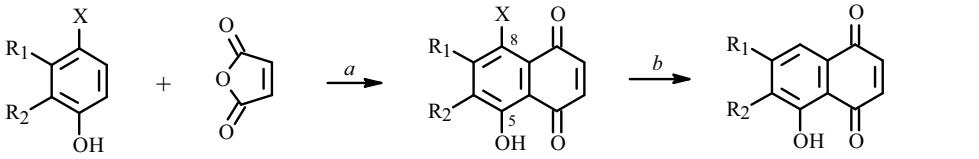
TABLE 1. IC_{50}, Cytotoxicity EC_{50} on Vero Cell, and HIV-1 Reverse Transcriptase (RT) Inhibition (%) Activity of Synthesized 1,4-Naphthoquinones at Different Concentrations

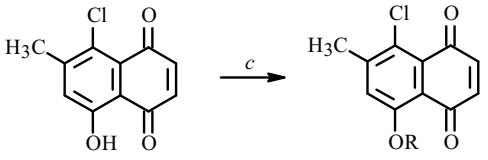
Compound	IC_{50}, μg/mL	EC_{50}, μg/mL	SI	Concentration, μg/mL			
				100	50	25	12.5
1	13.0	7.6	0.58	**100**	**85**	63	49
2	50.0	2.5	0.05	**95**	56	Nt.	Nt.
3	48.0	3.6	0.07	70	51	Nt.	Nt.
4	12.5	4.6	0.36	**97**	**68**	67	52
5	**6.0**	15.1	2.51	**100**	**98**	**94**	**80**
6	23.0	3.1	0.13	**100**	**75**	56	Nt.
7	25.0	7.7	0.30	**94**	**70**	50	Nt.
8	22.0	36.1	1.64	**80**	**60**	55	Nt.
9	> 100.0	Nt.	–	29	Nt.	Nt.	Nt.
10	> 100.0	Nt.	–	0	Nt.	Nt.	Nt.
11	> 100.0	Nt.	–	10	Nt.	Nt.	Nt.
12	> 100.0	Nt.	–	17	Nt.	Nt.	Nt.
Doxorubicin	47.0			98	53	Nt.	Nt.

SI: selective index (EC_{50}/IC_{50}). Nt.: not tested.



1 - 4 **5, 6**

1: $R_1 = CH_3$, $R_2 = H$, X = F; **2:** $R_1 = CH_3$, $R_2 = H$, X = Cl
3: $R_1 = CH_3$, $R_2 = H$, X = Br; **4:** $R_1 = H$, $R_2 = CH_3$, X = Cl
5: $R_1 = CH_3$, $R_2 = H$; **6:** $R_1 = H$, $R_2 = CH_3$



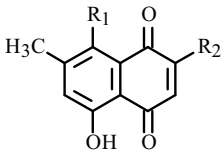
2 **7, 8** **7:** $R = CH_3$; **8:** $R = C_2H_5$

a. $AlCl_3$, 180°C, NaCl; *b*. $SnCl_2$/HCl, THF, 60°C, stirring; *c*. RI, Ag_2O, acetone.

Current treatment relies on a multiple drug combination regime HAART [10], which uses drugs targeting at least two viral proteins, and are very expensive. Hence, there is continuous research to develop new inhibitors with novel structures that act at different sites on HIV-RT and possess different mechanisms of action, or that act at other key viral proteins on enzymes. These can be added to current HIV-therapy for broader, safer, and cheaper medications for effective control of HIV infection.

1,4-Naphthoquinones are an important class of compounds due to their wide range of applications as pharmaceutical agents such as antitumor, antiproliferative, anti-inflammatory, antimycobacterial, antimalarial, antiviral, anticancer, and antileishmanial agents [11]. Some naphthoquinones, such as 1,4-naphthoquinone, vitamin K, juglone, plumbagin, diterpenoid quinones, and tanshinones moderately inhibits RNase H [12]. But the potential of this class as antivirals against HIV is poorly described in the literature. Incidentally doxorubicin, the anti-HIV drug, and its analogues anthracyclines, belong to the quinonoid family, which are ubiquitous secondary metabolites of living systems where they play essential roles in the biochemistry of energy production [13].

Streptonigrin is a good inhibitor of HIV-1 RT [14]. A comparative study of the effectiveness of some naphthoquinones (1,4-naphthoquinone, 5-hydroxy-1,4-naphthoquinone, 2,3-dichloro-1,4-naphthoquinone, and 2-methyl-1,4-naphthoquinone) against mammalian DNA polymerases (α & β), HIV-RT, and Avian myeloblastosis virus reverse transcriptase (AMV-RT) has been reported [15]. The fact that these compounds with significant activity against both HIV and AMV RT is suggestive of a mode of binding to HIV-1 RT distinct from that of the other non-nucleoside inhibitors since NNI′s in general do not inhibit AMV RT. Thus exploration of the "quinone-binding' site may eventually yield new classes of anti-HIV agents.



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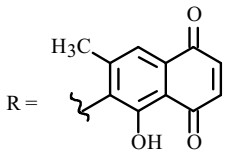
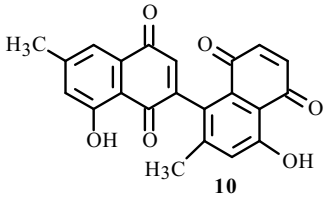
9: R_1 = H, R_2 = R

11: R_1 = R, R_2 = H

R =





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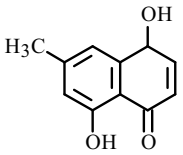

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In order to identify potent lead compounds against HIV-1 RT, our research efforts on discovery and development have been directed towards generating new chemical entities, which can be effective antivirals with low toxicity. In the present study, the synthesis and antiHIV-1 reverse transcriptase inhibition activity of 5-hydroxy-1,4-naphthoquinone analogues and some of their dimers were carried out.

The 8-fluoronapthoquinone **1** was prepared by the reaction of 4-fluoro-3-methylphenol with maleic anhydride under Friedel-Crafts acylation conditions. Compounds **2–4** were prepared in a similar manner. Reduction of the chlorine substituent of **2** and **4** was achieved by treatment with tin (II) chloride to access **5** and **6** [16]. The 5-*O*-alkylated derivatives **7**, **8** of **2** were generated by treating with the corresponding alkyl iodide, Ag_2O in acetone at 60°C, for 2–4 h with 96–98% yield.

Compounds **9–12** were isolated from the chloroform extract of *Euclea natalensis* roots, and separated and purified by column chromatography [17].

All the synthesized and isolated compounds were characterized by spectroscopic (UV, IR, NMR, and MS) and elemental analysis. Subsequently, the 1,4-naphthoquinones **1–12** were screened for their antiHIV-1 RT activity (% inhibition), and IC_{50} values were calculated using a non-radioactive HIV-RT colorimetric ELISA kit from Roche Diagnostic, Germany.

As indicated in Table 1, all the synthesized 1,4-naphthoquinone compounds exhibited potent inhibitory activity (70–100%) at 100 μg/mL.

Among these, compounds **5**, **1**, and **6** showed highly potent inhibition with 98%, 85%, and 75% and IC_{50} 6.0, 13.0, and 23.0, respectively, at 50 μg/mL, whereas doxorubicin shows 53% with IC_{50} 47.0. Especially, 5-hydroxy-7-methyl-1,4-naphthoquinone (**5**) was found to exhibit exceptionally potent and promising antiHIV-1 reverse transcriptase inhibition (80%) activity even at 12.5 μg/mL. From the comparative studies, it is possible to draw some structure– activity relationships. The activity results show that the inhibitory potency is decreased when the methyl group is shifted from C-7 of compound **5** to C-6 of compound **6**. However, the increase in the inhibition potency was noticed in the case of the corresponding 8-chloro compounds (**4** > **2**), which can be attributed to the different positions of the methyl group, i.e., ortho- and meta- to chlorine. Comparing the activities of **1–3** with halides at C-8, we found that the inhibition increase with increase in halide bulkiness and decrease in halide electronegativity.The 8-chloro (**2**, **4**) and 5-*O*-alkyl (7, **8**) derivatives, though less active than **5** and **6**, were comparable with doxorubicin (95%, 97%, 94%, and 80%, respectively, at 100 μg/mL). The results indicate that the derivatization of the 5-hydroxy group of compound **2** (95%, IC_{50} 50.0) to alkoxy, **7** and **8** (94% and 80%, IC_{50} 25.0 and 22.0 respectively), decreased the activity.

Surprisingly, the dimers **9–11** isolated were found to show very weak or no activity. This can be attributed to the structures of the dimers, where the C-2 or C-3 of the quinone ring of one molecule are connected to the aryl ring of the other, while in the case of compound **12** it may be due to lack of the quinone motif.

Cytotoxicity results for the Vero cell line indicate that the least toxic compound (EC_{50} 15.1 μg/mL) with good anti-HIV activity is **5** (Table 1).

In conclusion, a series of 1,4-naphthoquinone compounds was synthesized and evaluated for their HIV-1 reverse transcriptase inhibition activity. While 5-hydroxy-7-methyl-1,4-naphthoquinone (7-methyljuglone) was found to have exceptionally potent activity, its modified derivatives have also demonstrated significant activity. The promising moieties can be developed as new antiHIV-1 RT inhibitors by further structural modification.

EXPERIMENTAL

Chemicals and solvents used were purchased from Sigma–Aldrich Chemicals. Reactions were monitored using Merck silica gel 60 alumina sheets. Purification of compounds was done using column chromatography (silica gel 60–120 mesh). Melting points were determined on a Buchi apparatus and are uncorrected. The IR spectra were recorded on a Perkin–Elmer

Spectrum RXI FT-IR spectrometer. ^1H and ^{13}C NMR spectra were measured in $CDCl_3$ with a Varian 200 MHz NMR spectrometer with chemical shift values represented as δ (ppm) relative to the internal standard TMS. High-resolution EI mass spectra were obtained using a JEOL JMS–AX505 double-focusing mass spectrometer. The non-radioactive HIV-RT colorimetric ELISA kit was obtained from Roche Diagnostic, Germany.

General Procedure for the Preparation of 8-Halogen Derivatives 1–4. A mixture of anhydrous $AlCl_3$ (40 g, 300 mmol) and NaCl (8 g, 137 mmol) was heated to 180°C. A mixture of the appropriate 4-halo-3-methylphenol (2 g, 10.7 mmol) or 4-halo-2-methylphenol and maleic anhydride (4 g, 40.8 mmol) was added to the above melt with vigorous stirring for 2 min, and then poured into a mixture of ice and 12 M HCl. The mixture was kept for 30 min, and the precipitate was filtered and dried at room temperature overnight. The residue obtained was powdered and extracted with *n*-hexane with vigorous stirring at 50°C. The extract was concentrated under reduced pressure and crystallized from chloroform to afford the corresponding halogenated products.

8-Fluoro-5-hydroxy-7-methyl-1,4-naphthoquinone (1). Obtained as dark orange needles from $CHCl_3$, yield 25%, mp 150°C. IR (KBr, cm^{-1}): 1663, 1642 (C=O). ^1H NMR (200 MHz, $CDCl_3$, δ, ppm): 12.59 (1H, s, 5-OH), 7.31 (1H, s, H-6), 6.95 (2H, s, H-2, H-3), 2.53 (3H, s, CH_3). ^{13}C NMR ($CDCl_3$, 50 MHz, δ) 189.51 (C-4), 183.22 (C-1), 160.54 (C-5), 148.99 (C-8), 140.85 (C-2), 140.36 (C-3), 137.61 (C-7), 136.62 (C-9), 125.89 (C-6), 114.53 (C-10), 21.89 (C-11). HR-EI-MS *m/z* 206.0372 (calcd for $C_{11}H_7FO_3$ 206.0379).

8-Chloro-5-hydroxy-7-methyl-1,4-naphthoquinone (2). Obtained as dark orange shining needles from $CHCl_3$, yield 30%, mp 159°C. IR (KBr, cm^{-1}): 1662, 1646 (C=O). ^1H NMR (200 MHz, $CDCl_3$, δ, ppm): 12.54 (1H, s, 5-OH), 7.18 (1H, s, H-6), 6.88 (2H, s, H-2, H-3), 2.46 (3H, s, CH_3). HR-EI-MS *m/z* 222.0088 (calcd for $C_{11}H_7ClO_3$ 222.0084).

8-Bromo-5-hydroxy-7-methyl-1,4-naphthoquinone (3). Obtained as dark red needles from $CHCl_3$, yield 27%, mp 154°C. IR (KBr, cm^{-1}): 1663, 1642 (C=O). ^1H NMR (200 MHz, $CDCl_3$, δ, ppm): 12.54 (1H, s, 5-OH), 7.18 (1H, s, H-6), 6.88 (2H, s, H-2, H-3), 2.47 (3H, s, CH_3). ^{13}C NMR (50 MHz, $CDCl_3$, δ): 189.50 (C-4), 183.21 (C-1), 160.54 (C-5), 148.99 (C-8), 140.84 (C-2), 140.69 (C-3), 136.61 (C-7), 136.52 (C-9), 125.88 (C-6), 114.53 (C-10), 21.89 (C-11). HR-EI-MS *m/z* 265.9574 (calcd for $C_{11}H_7BrO_3$ 265.9579).

8-Chloro-5-hydroxy-6-methyl-1,4-naphthoquinone (4). Obtained as dark orange shining needles from $CHCl_3$, yield 30%, mp 162°C. IR (KBr, cm^{-1}): 1663, 1655 (C=O). ^1H NMR (200 MHz, $CDCl_3$, δ, ppm): 12.99 (1H, s, 5-OH), 7.48 (1H, s, H-7), 6.89 (2H, s, H-2, H-3), 2.32 (3H, s, CH_3).

General Procedure for the Preparation of 5-Hydroxy-7- and 6-methyl-1,4-naphthoquinones (5 and 6). A solution of the appropriate 8-chloro-1,4-naphthoquinones (200 mg, 0.90 mmol) in THF (20 mL) was added dropwise to a solution of $SnCl_2$ (1.0 g, 51 mmol) in 4 M HCl (70 mL) and THF (20 mL) at 60°C and stirred for 3 h. It was then cooled and filtered into a solution of $FeCl_3$. The resulting precipitate was filtered and dried to afford the required products.

5-Hydroxy-7-methyl-1,4-naphthoquinone (5). Obtained as orange needles from $CHCl_3$, yield 65%, mp 125°C. IR (KBr, cm^{-1}): 1670, 1645 (C=O). ^1H NMR (200 MHz, $CDCl_3$, δ, ppm): 11.84 (1H, s, 5-OH), 7.42 (1H, s, H-8), 7.06 (1H, s, H-6), 6.89 (2H, s, H-2, H-3), 2.41 (3H, s, CH_3). HR-EI-MS *m/z* 188.0475 (calcd for $C_{11}H_8O_3$ 188.0473).

5-Hydroxy-6-methyl-1,4-naphthoquinone (6). Obtained as dark orange needles from $CHCl_3$, yield 60%, mp 104°C. IR (KBr, cm^{-1}): 1663, 1655 (C=O). ^1H NMR (200 MHz, $CDCl_3$, δ, ppm): 13.0 (1H, s, 5-OH), 7.48 (1H, s, H-7), 7.23 (1H, s, H-8), 6.89 (2H, s, H-2, H-3), 2.32 (3H, s, CH_3). ^{13}C NMR (50 MHz, $CDCl_3$, δ): 15.81 (CH_3), 115.00, 124.64, 126.04, 136.60, 136.76, 140.24, 140.94, 160.22 (C-5), 182.62 (C=O), 190.10 (C=O). HR-EI-MS *m/z* 188.0462 (calcd for $C_{11}H_8O_3$ 188.0473).

General Procedure for the Preparation of 5-Alkoxyderivatives 7 and 8. A mixture of compound **2** 100 mg, Ag_2O (130 mg, 0.56 mmol), and either methyl- or ethyl iodide (48.19 mmol) in acetone (3 mL) was refluxed at 60°C for 2–4 h. The reaction mixture was then filtered and concentrated under reduced pressure. It was purified by silica gel chromatography and then crystallized (from hexane–chloroform) to afford the respective 5-methoxy- and 5-ethoxy- 1,4-naphthoquinone derivatives.

8-Chloro-5-methoxy-7-methyl-1,4-naphthoquinone (**7**). Obtained as yellow amorphous powder, yield 70%, mp 142°C. IR (KBr, ν_{max}, cm^{-1}): 1657 (C=O). ^1H NMR (200 MHz, $CDCl_3$, δ, ppm): 7.28 (1H, s, H-6), 6.85 (1H, s, H-3), 6.83 (1H, s, H-2), 4.02 (3H, s, OCH_3), 2.56 (3H, s, CH_3). HR-EI-MS *m/z* 236.0252 (calcd for $C_{12}H_9ClO_3$ 236.0240).

8-Chloro-5-ethoxy-7-methyl-1,4-naphthoquinone (8). Obtained as a brown semisolid, yield 70%. IR (KBr, ν_{max}, cm^{-1}): 1658 (C=O). ^1H NMR (200 MHz, $CDCl_3$, δ, ppm): 7.31 (1H, s, H-6), 6.85 (1H, s, H-3), 6.84 (1H, s, H-2), 4.24 (2H, q, J = 7.5, OCH_2), 2.56 (3H, s, 7-CH_3), 1.62 (3H, t, J = 7.5, CH_2CH_3). ^{13}C NMR (50 MHz, $CDCl_3$, δ): 185.58 (C=O), 183.92 (C=O), 159.18 (C-5), 146.17, 140.95, 140.94, 135.88, 133.70, 119.82, 119.43, 65.02 (OCH_2), 22.19 (CH_3), 14.61 (CH_3). HR-EI-MS *m/z* 250.0137 (calcd for $C_{13}H_{11}ClO_3$ 250.0397).

Isolation of Dimers. Compounds **9–12** were isolated from the chloroform extract of *Euclea natalensis* roots and identified by comparing the spectral data with the reported.

HIV-1 Reverse Transcriptase Assay. The standard reverse transcriptase assay is a specific, sensitive, simple, and reliable method for discovering anti-HIV agents. Both the synthesized and isolated compounds were evaluated for their inhibitory activity against recombinant HIV-1 enzyme *in vitro* using a non-radioactive HIV-RT colorimetric ELISA kit from Roche Diagnostic, Germany [18, 19]. The protocol outlined in the kit was followed using 2 ng of enzyme in a well and incubating the reaction for 2 h at 37°C. The compounds were screened at 100, 50, 25, and 12.5 μg/mL concentrations. Compounds which inhibit 50% or less were not tested further. Doxorubicin was used as a positive control. The assay was carried out in triplicate.

Cytotoxicity Assay. Vero cells were maintained in culture flasks in complete Eagle's MEM (minimum essential medium). Subculture was done every 2–3 days after it had formed a confluent monolayer. During subculture, cells that attached to the culture flask were trypsinized (0.25% trypsin containing 0.01% EDTA) for 10 min at 37°C, then stopped by the addition of complete medium. About 10^5 of the viable cells were then resuspended in complete medium.

Cytotoxicity was measured by the XTT (sodium 3′-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis-[4-methoxy-6-nitro)benzene sulfonic acid hydrate) method using the Cell Proliferation Kit II (Roche Diagnostics GmbH). The Vero cells (100 μL) were seeded at 1×10^5 onto a microtiter plate and incubated for 24 h to allow the cells to attach to the bottom of the plate. A dilution series was made of the derivatives (final concentration 200–0.2 μg/mL), added to the microtiter plate, and incubated for 48 h. The XTT reagent was added to a final concentration of 0.3 mg/mL, and the whole was incubated for 1–2 h. After incubation the absorbance of the color complex was quantified at 490 nm using an ELISA plate reader with the reference wavelength set at 690 nm. Fifty percent inhibitory concentration (EC_{50}) was defined as the concentration of the compounds at which absorbance was reduced by 50%. Compounds that showed IC_{50} greater than 100 μg/mL were not screened for cytotoxicity.

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