Hexaprenoid Hydroquinones, Novel Inhibitors of the Reverse Transcriptase of Human Immunodeficiency Virus Type 1

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HEXAPRENOID HYDROQUINONES, NOVEL INHIBITORS OF THE REVERSE TRANSCRIPTASE OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1

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ABSTRACT.—Activity against human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) in the organic extract of the Red Sea sponge *Toxiclona toxius* was traced by us to five novel natural compounds, namely toxiusol [**1**], shaagrockol B [**3**], shaagrockol C [**4**], toxicol A [**6**], all of which are sulfated hexaprenoid hydroquinones, and toxicol B [**7**], the *p*-hydroquinone derivative of compound **6**. The hydrolysis of the two sulfated compounds **1** and **4** yielded the corresponding hydroquinones designated as compounds **2** and **5**, and further oxidation of compound **7** afforded the corresponding *p*-quinone derivative, compound **8**. All compounds exhibited inhibitory activity of both DNA polymerizing functions of HIV-1 RT but failed to inhibit the RT-associated ribonuclease H activity. Toxiusol [**1**] was found to be the most potent inhibitor of the RNA-dependent DNA polymerase function (with 50% inhibition obtained at 1.5 μM and 95% inhibition at 4.6 μM), whereas the DNA-dependent DNA polymerase was significantly less sensitive to the inhibitor (with 50% inhibition achieved at 6.6 μM and 95% inhibition only at 41.6 μM). The fact that compound **1** discriminates between the two DNA polymerase activities of the RT offers new prospects for developing potent and highly specific anti-RT compounds, since the RNA-dependent DNA polymerase activity of RT is the only unique function that is not expressed at significant levels in uninfected mammalian cells.

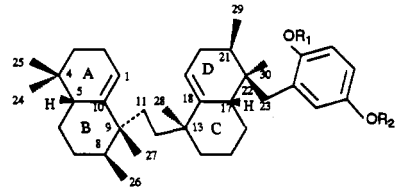
Reverse transcriptase (RT), a key enzyme in the life cycle of human immunodeficiency virus (HIV), was found to be so far the most successful target for the chemotherapeutic treatment of acquired immunodeficiency syndrome (AIDS) (1). RT is a multifunctional enzyme exhibiting two DNA polymerase activities, RNA-dependent DNA polymerase (RDDP) and DNA-dependent DNA polymerase (DDDP), as well as an inherent ribonuclease H (RNase H) activity (2,3). Inhibition of each of the catalytic functions interferes with the virus production in the host cells. Nucleoside analogues, which serve as chain terminators of DNA synthesis, including 3′-azidothymidine (AZT) and dideoxyinosine (ddI), are so far the only approved drugs for treatment of HIV infections (4,5). Unfortunately, their use is limited due to the rapid emergence of resistant viral strains (6,7) and to the toxic side effects (8), which may reflect inhibition of cellular DNA polymerases. Other potent RT inhibitors such as suramin (9) and HPA23 (10) turned out also too toxic to be considered as potential anti-HIV drugs. The relatively new nonnucleoside inhibitors of HIV-1 RT, which belong to a structurally unrelated pharmacological class of compounds (11,12), have been demonstrated to be the most potent and selective agents against HIV-1 RT. At least four of these nonnucleoside inhibitors have been progressed into phase-II clinical trials (11). Unfortunately, the initial findings are somewhat pessimistic. The fact that rapid drug resistance was observed in patients after 6–12 weeks of therapy has led to the current opinion that these agents are unsuitable for chemotherapy. Consequently, the urgent need for the development of effective and selective drugs against AIDS still remains.

Marine natural products represent a potential source for RT inhibitors. Substances isolated from the Red Sea marine organisms, such as avarol analogues, illimaquinone,

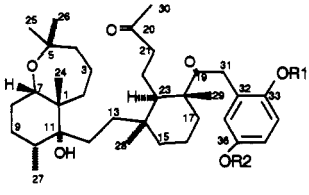
and halocynthiaxanthin, were also reported by us to inhibit effectively the reverse transcriptase of HIV (13–16). In view of these encouraging findings we have decided to continue screening for additional marine metabolites of diverse marine Red Sea fauna for their anti-HIV RT activities. In the present study evidence is provided that five new hexaprenoid hydroquinones, namely toxicols A [**6**] and B [**7**], toxiusol [**1**], and shaagrockols B [**3**] and C [**4**], which were isolated from the Red Sea sponge *Toxiclona toxius* Levi (Callyspongiidae), are potent inhibitors of HIV-1 RT.

RESULTS AND DISCUSSION

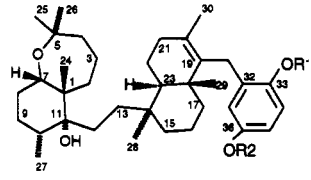
BIOASSAY-GUIDED ISOLATION OF ANTI-HIV-1 RT CONSTITUENTS.—In our continuing search for marine metabolites with potent anti-HIV RT activity we have found that the MeOH-CH_2Cl_2 (1:1) extract of the sponge *T. toxius* inhibits the HIV-1 RT associated RDDP activity at a concentration of 10 μg/ml by 97%. An initial solvent-solvent partition of the crude extract (between aqueous MeOH against petroleum ether and CCl_4 distributed the total anti-HIV RT bioactivity in the aqueous MeOH portion. This fraction was found to effectively inhibit the RDDP activity by 100% at a concentration of 10 μg/ml extract. Chromatography of the aqueous MeOH portion by repeated reversed-phase vlc and hplc yielded five compounds: toxiusol [**1**], shaagrockols B [**3**] and C [**4**], and toxicol A [**6**], all of which are sulfated hexaprenoid hydroquinones, and toxicol B [**7**], a *p*-hydroquinone derivative of compound **6**. Other related compounds were prepared as described in the Experimental. Initial screening for the inhibitory activity of the poly $(rA)_n$·oligo $(dT)_{12\text{-}18}$-directed synthesis of HIV-1 RT revealed that all the compounds inhibit more than 50% of the initial RDDP function at a final inhibitor concentration of 10 μg/ml.



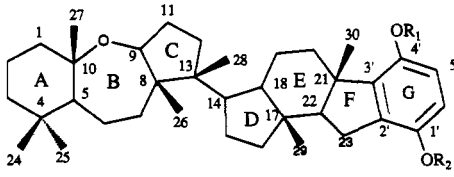
1 $R_1=R_2=SO_3^-Na^+$
2 $R_1=R_2=H$



3 $R_1=R_2=SO_3^-Na^+$



4 $R_1=R_2=SO_3^-Na^+$
5 $R_1=R_2=H$



6 $R_1=R_2=SO_3^-Na^+$
7 $R_1=R_2=H$
8 $R_1=R_2=O$=

EFFECT OF THE HEXAPRENOID HYDROQUINONE SULFATES ON HIV-1 RT.—Compounds that inhibit in vitro the catalytic activities of HIV RT are likely to fall into one of three categories: (I) compounds that block all three catalytic activites, i.e., RDDP, DDDP, and the RNase H activity associated with the reverse transcriptase; (II) inhibitors of the DNA polymerase activity with little or no effect on the RNase H activity; or (III) compounds that block the RNase H activity without significantly affecting the DNA polymerase function. The inhibitory effect of the natural sulfated hexaprenoids **1**, **3**, **4**, and **6** on the three functions associated with HIV-1 RT were evaluated and were found to fall into the second category. We have assayed the two DNA polymerase activities in the presence of increasing concentrations of each compound and the RNase H activity at a final inhibitor concentration at 250 μM. The calculated 50% inhibitory concentrations (IC_{50} values) [as determined from the dose-dependent curves (Figure 1)] for the RT-associated RDDP function were 1.5±0.2 μM, 8.5±0.1 μM, 3.3±0.7 μM, and 3.1±0.8 μM for toxiusol [**1**], shaagrockol B [**3**], shaagrockol C [**4**], and toxicol A [**6**], respectively. The IC_{50} values for the activated DNA-directed synthesis of compounds **1**, **3**, **4**, and **6** were 6.6±1.9 μM, 6.7±0.9 μM, 0.8±0.0 μM, and 2.7±0.0 μM, respectively. All four compounds were devoid of any significant activity against the RNase H activity. The insensitivity of the RNase H activity is such that even at a concentration of 250 μM of compounds **1**, **3**, **4**, and **6**, the extent of inhibition is only about 15%, 1%, 5%, and 28%, respectively (IC_{50} values >250 μM; see Table 1). Of the four compounds tested, shaagrockol B [**3**] exhibited only a moderate activity against the DNA polymerase function, whereas compounds **4** and **6** inhibited effectively both RT-associated DNA polymerase activities. Toxiusol [**1**] exhibited lower inhibitory capacity towards the DDDP activity, with 50% inhibition achieved at 6.6±1.9 μM and 95% inhibition only at 41.6±2.8 μM. This means that the RNA-directed synthesis is about fourfold more sensitive to compound **1** than is the activated DNA-directed synthesis. The fact that compound **1** discriminates between the two DNA polymerase activities of the HIV-1 RT offers new prospects for developing potent and highly specific anti-RT compounds, because the RDDP activity is the only unique activity of RT that is not expressed by enzymes present in mammalian cells. Compound **3**, on the other hand, shows a higher potency in inhibiting the DDDP activity than in inhibiting the RDDP; i.e., only 0.8±0.0 μM, compared to the 3.3±0.7 μM of this inhibitor was required to inhibit half of the initial activity of the HIV-1 RT-associated DDDP and RDDP activities, respectively.

The inhibitory effects of compounds **2**, **5**, **7**, and **8**, all of which are derivatives of the hexaprenoid hydroquinone sulfates, are summarized in Table 1. All compounds were found to be potent inhibitors of both DNA polymerase activities but failed to inhibit the HIV-1 RT-associated RNase H activity. In general, hydrolysis of the two sulfates to the corresponding hydroquinones does not affect substantially their inhibitory potential. However, the *p*-quinone derivative **8** of toxicols A and B (where the sulfates and hydroxyl groups were substituted to yield a quinone form) demonstrates a reduced inhibitory activity of the RNA-directed synthesis; i.e., 50% and 95% inhibition is achieved only at inhibitor concentrations of 13.2±0.0 μM and 28.6±0.4 μM, respectively. In short, we predict that the negatively charged sulfate moieties or the hydroxyl groups might interact with positively charged side chains on the protein that are associated with its catalytic sites. Various other sulfonic acid derivatives have been shown to exhibit anti-HIV RT activity, such as naphthalenesulfonic acid (17), surmain (9), and its sulfonic acid analogues (18). We have shown that the hydroxyl group at the ortho position to the carbonyl group in the quinone ring of the natural products avarol analogues and illimaquinone, is a requisite for the anti HIV-1 RT inhibitory activity of these

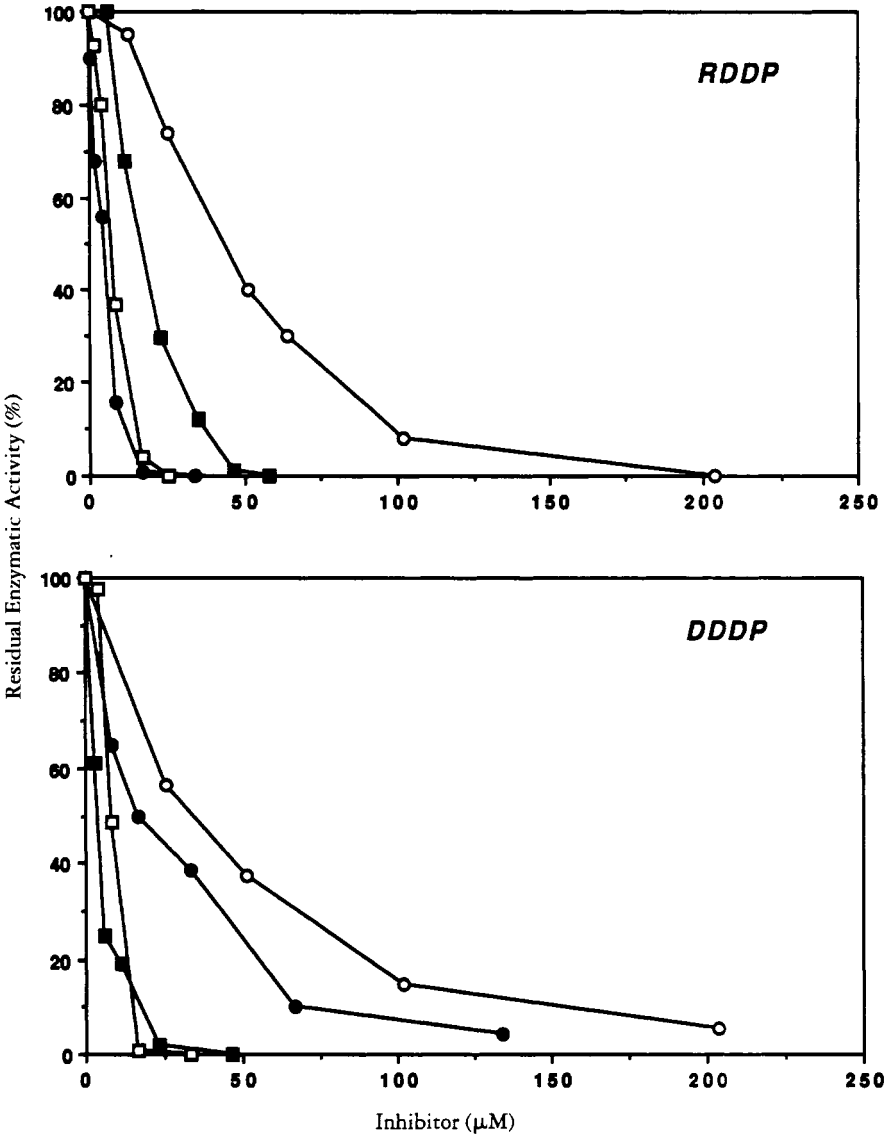


FIGURE 1. Dose-response curves of the inhibition of the two DNA-polymerase activities of HIV-1 RT. Increasing amounts of the four hexaprenoid hydroquinone sulfates were added to HIV-1 RT and after 5 min. preincubation at 30° the RDDP and DDDP activities were assayed as described under "Experimental." The residual enzymatic activities were calculated as percentage of control enzyme with no inhibitor. Compound **1** (●); compound **3** (○); compound **4** (■); compound **6** (□).

compounds (13,14). Similarly, we have demonstrated that the hydroxyl group in the ortho position to the carbonyl group in the pyridinone ring of the natural compound 3,5,8-trihydroxy-4-quinolone is probably the key minimal structural requirement for its inhibitory activity (19).

The present results indicate that the new natural products, the hexaprenoid hydroquinone sulfates series, can be considered novel leads for the development of new potent and selective anti-AIDS agents.

TABLE 1. Inhibition of HIV-1 Reverse Transcriptase by Hexaprenoid Hydroquinone Sulfates and Their Derivatives.

Compound	RDDP		DDDP		RNase H
	IC_{50}	IC_{95}	IC_{50}	IC_{95}	IC_{50}
1	1.5±0.2	4.6±0.1	6.6±1.9	41.6±2.8	>250
2	2.8±0.09	11.1±2.5	4.2±0.4	44.4±5.8	>>250
3	8.5±0.1	21.2±1.7	6.7±0.9	40.0±0.0	>>250
4	3.3±0.7	9.3±0.2	0.8±0.0	5.0±0.0	>>250
5	2.5±0.7	18.1±3.6	1.4±0.3	14.7±0.9	>>250
6	3.1±0.8	6.5±1.1	2.7±0.0	5.2±0.0	>>250
7	3.7±0.8	11.1±4.2	8.2±1.1	56.2±3.7	>>250
8	13.2±0.0	28.6±0.4	4.5±1.1	54.5±5.5	>250

[a]The IC_{50} and IC_{95} values (the inhibitor concentrations that lead to 50% or 95% inhibitions respectively, of the initial enzymatic activities) are expressed in μM of each compound. All data represent mean values (± range) for at least two separate experiments. RDDP=RNA-dependent DNA polymerase; DDDP=DNA-dependent DNA polymerase; RNase H=inherent ribonuclease H.

EXPERIMENTAL

MATERIALS.—The five new compounds toxiusol [**1**], shaggrockol B [**3**], shaagrockol C [**4**], toxicol A [**6**], and toxicol B [**7**] have been isolated from the Red Sea sponge *T. toxius* and their structures identified as described previously (20,21). The derivatives **2**, **5**, and **8** were prepared as described (21). Compounds **2** and **5** are the corresponding *p*-hydroquinones of the natural sulfated compounds **1** and **4**, respectively. Compound **8** is the *p*-quinone derivative of the natural product toxicol A. [^{3}H]dTTP and [^{3}H]poly(rA)$_n$ were purchased from Amersham. Synthetic primer-template poly r(A)$_n$·oligo (dT)$_{12-18}$ and the poly (dT)$_n$ were the products of Pharmacia. Activated gapped DNA was prepared by a limited digestion of salmon sperm DNA with bovine pancreatic DNase-I as described (22).

ENZYMES.—The reverse transcriptase of HIV-1 used in this study was recombinant enzyme expressed by us in *Escherichia coli*. HIV-1 RT was derived from BH-10 proviral clone of HIV-1 (23). The enzyme was purified to near homogeneity according to the protocol described previously (24). This enzyme is the p66/p51 heterodimer, composed of two subunits with molecular weights of approximately 66 kDa and 51 kDa.

ENZYMATIC ASSAYS.—The RT-associated DNA polymerase and the RNase H activities were assayed as described previously (15,25). In short, the RDDP activities were assayed by monitoring the poly (rA)$_n$·oligo (dT)$_{12-18}$-directed incorporation of [^{3}H]dTTP into trichloroacetic-acid-insoluble DNA product. The DDDP activities were assayed with an activated DNA as primer-template and with all four deoxynucleotides present (of which only one, dTTP, was radioactively labeled). The RNase H activity was assayed by measuring the release of trichloroacetic-acid-soluble material from the synthetic substrate [^{3}H]poly(rA)$_n$·poly(dT)$_n$·This substrate was prepared according to the procedure described previously (15).

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