Biological Effects of Prenylated Hydroquinones: Structure-Activity Relationship Studies in Antimicrobial, Brine Shrimp, and Fish Lethality Assays

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BIOLOGICAL EFFECTS OF PRENYLATED HYDROQUINONES: STRUCTURE-ACTIVITY RELATIONSHIP STUDIES IN ANTIMICROBIAL, BRINE SHRIMP, AND FISH LETHALITY ASSAYS

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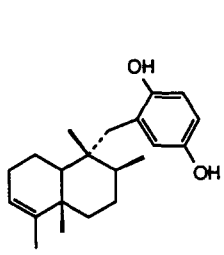
ABSTRACT.—Twenty-three hydroquinone and quinone derivatives were assayed for antimicrobial effects and brine shrimp and fish lethalities, to establish relevant structure-activity relationships (SAR). Linear 2-prenyl-1,4-hydroquinones used for bioassay were obtained either by isolation from the sponge *Ircinia spinosula* or by synthesis. Corresponding quinones, as well as hydroquinones possessing saturated side-chains composed of one to eight isopentane units, were also synthesized and biologically evaluated. Terpenoid 1,4-benzoquinones displayed moderate antimicrobial activity against three microorganisms. SAR studies indicate the optimum length of the side-chain is in the range of five to fifteen carbon atoms.

Terpenoid 1,4-benzoquinones such as tocopherols, ubiquinones, and plastoquinones are widespread in nature, and they play an important role in photosynthesis and electron transport (1,2). Unsubstituted prenylated benzoquinones/hydroquinones are common in marine organisms but have rarely been discovered in other organisms.

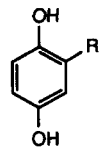
Several naturally occurring linear or cyclic prenyl-hydroquinones have been described to date with a terpenoid portion that ranges from one to eight isoprene units. Triprenyl- and tetraprenyl-hydroquinones are the most abundant compounds among this class of metabolites. Biological activities have been reported for relatively few polyprenyl-hydroquinones (3). Diprenyl- and triprenyl-hydroquinones are the most extensively studied, and they show a wide variety of biological activities (3). Among these compounds, avarol [**1**], a sesquiterpene hydroquinone possessing a rearranged drimane skeleton, and its quinone (avarone), previously isolated from the marine sponge *Dysidea avara* (4,5), are the most studied biologically. They show a wide variety of biological activities and low toxicity in mice (6), and in human testing revealed no side effects at an administered dose of 3 mg/kg (7). Both compounds are potent antileukemic agents in vitro (8) and in vivo (9). Recent biological studies on different derivatives of avarol and/or avarone, involving both the aromatic ring and sesquiterpenoid moiety of avarol, reveal that the terpenoid moiety plays a marginal role in biological processes, while the hydroquinone/quinone couple is the main group responsible for biological activity (10). These results and the lack of biological data on polyprenyl-hydroquinones prompted us to undertake a comprehensive study on the structure-activity relationships (SAR) among unsubstituted prenyl-hydroquinones.

In this paper, we report the acquisition of test compounds by isolation or synthesis and the determination of their SAR in antimicrobial, brine shrimp lethality, and mosquito fish lethality assays.

Linear 2-prenyl-hydroquinones [**4–9**] were synthesized or isolated from the sponge *Ircinia spinosula*. Prenyl-hydroquinones were, in general, synthesized by slowly adding the allylic alcohol to the acidic solution of hydroquinone, in anhydrous media. The synthesis of **5** was performed by modification of a previously reported method (11), using anhydrous conditions. The same method of synthesis was used to synthesize compound **4**. Compound **6**



1



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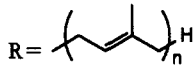
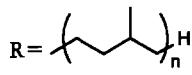
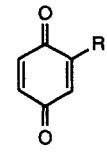
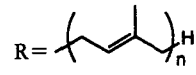




4	n=1	**18**	n=1
5	n=2	**19**	n=2
6	n=3	**20**	n=3
7	n=6	**21**	n=4
8	n=7	**22**	n=6
9	n=8	**23**	n=7
		24	n=8



10 R=H 11 R=Me



12	n=1	**16**	n=6
13	n=2	**17**	n=7
14	n=3	**18**	n=8
15	n=6		

was synthesized using the method previously described (12).

In order to obtain information on the comparative effects of corresponding prenyl-hydroquinone and prenyl-quinones, and the effect of flexibility of the side-chain, we synthesized the quinones **10–17** and the perhydroprenyl-hydroquinones **18–24** for bioassay.

Quinones **10–17** were obtained by oxidation of the corresponding hydroquinones with silver oxide, while the perhydro- derivatives **18–24** were obtained by hydrogenation. This resulted in three groups of derivatives, with each group containing side-chains of up to 40 carbons in length.

Preliminary results on antimicrobial effects were obtained using a disk diffusion test. The minimum inhibitory concentrations were determined only for those compounds that had previously shown an inhibition zone in the disk diffusion test; the results are reported in Table 1. None of the tested compounds showed effects against *E. coli* or *P. aeruginosa*, although members with a short side-chain (up to the triprenyl derivatives), within the three groups of derivatives, showed moderate effects against Gram-positive bacteria and fungi. The oxidation of the hydroquinone ring to quinone and the hydrogenation of the double bonds in the side-chain did not produce relevant variation in potency. The moderate potency of these compounds is in agreement with data previously reported for compound **5** (3,13), and for unsubstituted prenylated benzoquinones/hydroquinones (3,14).

Toxicity was determined by means of the brine shrimp (*A. salina*) lethality assay, which gives results that correlate well with cytotoxicity in cancer cell lines such as KB and P-388 (15), and L5178Y and L1210 (16). The results are reported in Table 1. In each group of derivatives, the most active was the representative with ten carbon atoms in the side-chain [**5**, **13**, **19**]. The monoprenyl-hydroquinone **4** showed an activity comparable to that of avarol [**1**], while di- and tri-prenyl derivatives [**5** and **6**] are more toxic than avarol. The trend was the same in each group of derivatives. In fact, the lethality increased with the number of carbon atoms until the diprenyl or triprenyl derivatives, then decreased with a further increase in the number of carbon atoms in the side-chain. Hydrogenation of the double bonds in the side-chain produced an increase in activity among derivatives with five to 20 carbon atoms [**18–21**], while a loss of activity was observed for derivatives with longer side-chains [**22–24**]. Thus, a higher degree of chain flexibility appears to be detrimental to the toxicity of derivatives that have more than 20 carbon atoms in their side-

TABLE 1. Biological Activities of Hydroquinone/Quinone Derivatives **1–24**.

Compound	Antimicrobial MIC (μg/ml)[a]			Brine shrimp assay[b] LC_{50} (95% C.L.)	Fish lethality[b] LC_{50} (95% C.L.)
	B. s.	*M. l.*	*C. a.*		
1	>50	>50	N.A.	0.18 (0.32/0.10)	4.6 (5.8/2.3)
2	>50	>50	N.A.	0.35 (1.25/0.16)	21.5 (41.9/11.1)
3	25	>50	>50	>10	38.5 (76.8/20.2)
4	>50	25	>50	0.54 (3.43/0.20)	22.2 (49.8/9.6)
5	6.25	12.5	25	0.01 (0.02/0.007)	4.6 (5.8/2.3)
6	1.56	12.5	N.A.	0.05 (0.08/0.03)	3.3 (4.8/2.2)
7	N.A.	N.A.	N.A.	0.63 (1.80/0.33)	>100
8	N.A.	N.A.	N.A.	0.91 (2.91/0.50)	>100
9	N.A.	N.A.	N.A.	0.98 (2.63/0.56)	>100
10	>50	>50	25	0.73 (1.78/0.38)	1.0 (4.1/0.24)
11	25	>50	>50	1.06 (2.61/0.55)	5.4 (6.6/3.8)
12	25	25	>50	0.19 (0.33/0.10)	1.4 (2.7/0.8)
13	12.5	12.5	25	0.05 (0.08/0.03)	0.2 (40.4/0.07)
14	>50	>50	N.A.	0.11 (0.20/0.05)	5.4 (6.6/3.8)
15	N.A.	N.A.	N.A.	1.08 (2.07/0.63)	>100
16	N.A.	N.A.	N.A.	1.26 (2.45/0.75)	>100
17	N.A.	N.A.	N.A.	1.45 (2.68/0.91)	>100
18	12.5	6.25	>50	0.29 (0.50/0.18)	3.3 (4.8/2.2)
19	6.25	6.25	N.A.	0.001 (0.002/0.0007)	0.15 (0.28/0.07)
20	>50	>50	N.A.	0.02 (0.04/0.01)	21.3 (38.8/11.7)
21	N.A.	N.A.	N.A.	0.02 (0.04/0.01)	43.6 (90.5/20.7)
22	N.A.	N.A.	N.A.	>10	>100
23	N.A.	N.A.	N.A.	>10	>100
24	N.A.	N.A.	N.A.	>10	>100
Gentamicin	0.78	1.56			
Miconazole			1.56		
Rotenone					0.03 (0.16/0.007)

[a] *B. s.* = *Bacillus subtilis*; *M. l.* = *Micrococcus luteus*; *C. a.* = *Candida albicans*. N.A. = no activity in the diffusion test.

[b] LC_{50} values are expressed in ppm; 95% C.L. = 95% confidence limits.

chain. Otherwise, a higher degree of side-chain flexibility may improve permeability across the cell membrane and produce an increase in toxic activity. Generally, the oxidation of the hydroquinone ring to quinone produced little loss in activity. In fact, no substantial difference was observed in the activities of **2–9** or **10–17**. An increase in activity was observed only for methyl-quinone [**11**] and monoprenyl-quinone [**12**] compared to the corresponding hydroquinones [**3** and **4**].

The organism used to determine fish lethality was the common mosquito fish *G. affinis*. This fish has been used successfully in previous lethality studies (17,18) and the results are in excellent agreement with lethality in mice (18). The results are given as LC_{50} (ppm) after 90 min exposure, and are reported in Table 1. In each group of derivatives the most potent was the compound with ten carbon atoms in the side-chain, while derivatives with longer side-chains did not show lethality at 100 ppm. The general trend was similar to that observed in the brine shrimp lethality assay. In fact, lethality increased until the derivative with ten carbon atoms in the side-chain, then decreased with further extension of the side-chain. In the group of prenyl-hydroquinones, only compounds **5** and **6** showed a lethality comparable to that of avarol, while the rest of the compounds were significantly less lethal than avarol [**1**]. Both the oxidation of the hydroquinone moiety to the quinone, and the hydrogenation of the side-chain produced an increase in lethal-

ity for derivatives with a shorter side-chain, showing the highest lethality for compounds with ten carbon atoms in the side-chain [**13** and **19**].

In summary, these results confirm the moderate antimicrobial effects of the terpenoid-hydroquinones/quinones (3) and clearly show that the optimum length of the side-chain, for potency in the assays used, is in the range of five to fifteen carbon atoms. The quinone derivatives are more toxic.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Hydroquinone, methylhydroquinone, 3-methyl-2-buten-1-ol, geraniol, farnesol, phytol, rotenone, miconazole, and gentamicin were purchased from Sigma-Aldrich s.r.l., Italy. Microorganisms were obtained from DSM, Germany. Avarol [**1**] was available in our laboratory; it was isolated from the marine sponge *Dysidea avara* as previously described (4). Synthetic and natural compounds [**4**–**24**] were identified by spectral data. Uv spectra were obtained with a Varian DMS 90 spectrophotometer. Mass spectra were recorded on a Fisons TRIO 2000 spectrometer, coupled with an INTEL computer. ^{1}H-Nmr spectra were recorded at 500 MHz, in $CDCl_3$ with TMS used as internal standard on a Bruker AM 500 instrument, under Aspect X32 control. Si gel chromatography was performed using pre-coated Merck F_{254} plates and Merck Kieselgel 60 powder. Prep. hplc purifications were carried out on a Waters apparatus equipped with a Spherisorb S5 ODS2 column (10 mm×25 cm) and with a refractive index detector.

BIOLOGICAL MATERIAL.—*Ircinia spinosula* Schulze (Dictyoceratida) was collected at about 15 m depth in the bay of Naples, and frozen at −20° until extracted. A voucher specimen has been deposited in the institute collection (Voucher No. SX/88).

EXTRACTION AND ISOLATION OF 2-HEXAPRENYL-HYDROQUINONE [**7**], 2-HEPTAPRENYL-HYDROQUINONE [**8**], AND 2-OCTAPRENYL-HYDROQUINONE [**9**].—The frozen sponge (90 g dry wt after extraction) was extracted with Me_2CO and, after elimination of the solvent *in vacuo*, the aqueous residue was extracted with Et_2O.

The Et_2O was evaporated *in vacuo* to obtain a brown oil (3 g), which was chromatographed on a Si gel column with a gradient system comprising petroleum ether (40–70°) and Et_2O to obtain a mixture of compounds **7**–**9** (600 mg) as previously described (19). The mixture was subjected to prep. hplc (*i*-PrOH-H_2O, 4:1) to obtain the pure compounds **7** (60 mg), **8** (320 mg), and **9** (180 mg). The identification of these isolates was accomplished by comparison of their spectral data (uv, ir, ms, nmr) with those previously reported (19).

SYNTHESIS OF 2-ISOPENTYL-HYDROQUINONE [**4**] AND 2-GERANYL-HYDROQUINONE [**5**].—To a mixture of 0.02 mole (2.2 g) hydroquinone and 350 mg of anhydrous oxalic acid in 6 ml of diglyme (2-methoxy ethyl ether) were slowly added 0.01 mole of 3-methyl-2-buten-1-ol (0.86 g) or geraniol (1.54 g). The mixtures, after 3 h at reflux, were extracted with Et_2O and chromatographed on a Si gel column with light petroleum ether-Et_2O (4:1), to obtain **4** and **5**, in yields of 30% and 25%, respectively. Identifications of compounds **4** and **5** were made on the basis of their spectral data (uv, ms, nmr) in comparison with published values (11,20).

SYNTHESIS OF 2-FARNESYL-HYDROQUINONE [**6**].—To a mixture of 0.02 mole of hydroquinone and 0.01 mole (2.2 g) of farnesol in 25 ml of anhydrous Et_2O were added 2 ml of BF_3 etherate. After 3 h under stirring at room temperature, H_2O was added, and the solution was extracted with Et_2O. The organic layer was chromatographed following the same procedure as for compounds **4** and **5**. Compound **6** (yield 35%) was identified by comparison of its spectral data (uv, ms, nmr) with those reported (21).

SYNTHESIS OF *p*-BENZOQUINONE [**10**], 2-METHYL-1,4-BENZOQUINONE [**11**], 2-ISOPENTYL-1,4-BENZOQUINONE [**12**], 2-GERANYL-1,4-BENZOQUINONE [**13**], 2-FARNESYL-1,4-BENZOQUINONE [**14**], 2-HEXAPRENYL-1,4-BENZOQUINONE [**15**], 2-HEPTAPRENYL-1,4-BENZOQUINONE [**16**], AND 2-OCTAPRENYL-1,4-BENZOQUINONE [**17**].—Hydroquinone [**2**], methylhydroquinone [**3**], and prenylhydroquinones **4**–**9** (20 mg each) were individually stirred with Ag_2O (20 mg) in Et_2O (5 ml) for 10 min at room temperature as described by Fieser (22). The mixtures were filtered and the solvent eliminated *in vacuo*, to give compounds **10**–**17** (yield 100% for each one). The identities of these compounds were confirmed by comparison of their spectral data (uv, ms, nmr) with those of corresponding hydroquinones.

SYNTHESIS OF 2-(3-METHYLBUTYL)HYDROQUINONE [**18**], 2-(3,7-DIMETHYLOCTYL)HYDROQUINONE [**19**], 2-(3,7,11-TRIMETHYLDODECYL)HYDROQUINONE [**20**], 2-(3,7,11,15,19,23-HEXAMETHYLTETRACOSYL)HYDROQUINONE [**22**], 2-(3,7,11,15,19,23,27-HEPTAMETHYLOCTACOSYL) HYDROQUINONE [**23**], AND 2-(3,7,11,15,19,23,27,31-OCTAMETHYLDOTRIACONTYL)HYDROQUINONE [**24**].—Solutions of prenylhydroquinones **4**–**9** (20 mg each) in MeOH (3 ml) containing 5% Pd on charcoal (10 mg) were stirred under 2.8 atmosphere of H_2 for 18 h, at room

temperature. After filtration, evaporation, and chromatography on a Si gel column (light petroleum ether-Et_2O, 4:1) perhydroprenyl-hydroquinones **18–20** and **22–24** were obtained (yield 95% for each compound). These compounds were identified by comparison of their spectral data (ms, nmr) with those of corresponding prenyl-hydroquinones.

SYNTHESIS OF 2-DIHYDROPHYTYL-HYDROQUINONE [**21**].—To a mixture of 0.02 mole of hydroquinone and 0.01 mole (2.96 g) of phytol in 25 ml of anhydrous Et_2O, were added 2 ml of BF_3 etherate, following the same procedure used for the synthesis of **6**. The phytyl-hydroquinone was reduced following the same procedure used for the synthesis of perhydroprenyl-hydroquinones, to afford **21** (yield 40%), which was identified by comparison of its spectral data (ms, nmr) with those of other perhydroprenyl-hydroquinones.

ANTIMICROBIAL AND ANTIFUNGAL ACTIVITIES.—Gram-positive bacteria [*Bacillus subtilis* (DSM 347), *Micrococcus luteus* (DSM 348) and *Pseudomonas aeruginosa* (DSM 1117)], the Gram-negative bacterium [*Escherichia coli* (DSM 1103)], and a fungus [*Candida albicans* (DSM 1665)] were used for the antimicrobial assays. Preliminary results for antibacterial and antifungal activities were obtained by means of diffusion tests, using 100 mg of compound per paper disk (6 mm). The impregnated disks were applied on nutrient agar plates, seeded with overnight cultures of the test microorganisms. The inhibition zones were measured after 18 h incubation at 35–37°.

Quantitative measurement on active compounds, selected by means of the diffusion test, were performed to determine the minimum inhibitory concentration (MIC). Twofold serial dilutions were tested, starting from a 50 μg/ml solution. The end point in this assay was indicated by the absence of detectable growth after 18 h of incubation at 35–37°.

BRINE SHRIMP LETHALITY.—The brine shrimp (*Artemia salina*) assay was performed in triplicate with appropriate amounts of compounds dissolved in DMSO (1% final volume) at concentrations of 10, 0.1, and 0.01 ppm, using 10 freshly hatched larvae suspended in 5 ml artificial sea water (15). Briefly, for each dose tested, surviving shrimp were counted after 24 h, and the data statistically analyzed by the Finney program (23), which affords LC_{50} values with 95% confidence intervals. Where data were insufficient for probit analysis, LC_{50} values were estimated using the results of assay at concentrations of 0.1, 0.01, 0.001, and 0.0001 ppm.

FISH LETHALITY.—A toxicity test in mosquito fish, *Gambusia affinis* (Baird and Girard), was performed in triplicate using 6 fish, 0.1 to 0.3 g in weight, placed in 70 ml of distilled H_2O to which appropriate amounts of compounds, dissolved in Me_2CO (1% final volume) at concentrations of 100, 10, 1, 0.1, 0.01, and 0.001 ppm, were added in each test. Two replicate controls (containing distilled H_2O and 1% Me_2CO) were utilized in conjunction with each assay. For each dose tested, survivor fish were counted after 90 min and the data were statistically analyzed by the Finney program (23), which affords LC_{50} values with 95% confidence intervals.

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