Potential Antipsoriatic Avarol Derivatives as Antioxidants and Inhibitors of PGE_2 Generation and Proliferation in the HaCaT Cell Line

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The synthesis and structure−activity relationships for a series of 14 new avarol derivatives as antioxidants and inhibitors of cell proliferation and PGE_2 generation in human keratinocytes are described. Compound **6** (thiosalicylic derivative) was the most potent inhibitor of superoxide generation in human neutrophils and also potently inhibited PGE_2 generation in the human keratinocyte HaCaT cell line. Compound **7** (3′-methylaminoavarone) presented the best antiproliferative profile, by the inhibition of ^{3}H-thymidine incorporation in HaCaT cells, with potency similar to the reference compound anthralin. None of the avarol derivatives showed any sign of cytotoxicity measured as LDH release in treated keratinocytes. The potency and pharmacological profile of derivatives are also discussed.

Avarol (**1**) is a marine sesquiterpenoid hydroquinone possessing a rearranged drimane skeleton with interesting pharmacological properties including anti-inflammatory, antitumor, and antiviral actions.[1−3] Previous studies demonstrated the antioxidant properties of avarol, which inhibits superoxide generation and microsomal lipid peroxidation.[4,5] The biological activities of this compound have been correlated with its redox chemistry and its ability to effect radical production, while the terpenoid moiety plays a marginal role in biological processes.[6] These interesting properties and the previous findings that avarone, the quinone of avarol, reacts toward protein sulfhydryl groups,[7] and that 5′-monoacetyl and diacetyl avarol, from *D. avara*,[8] and some amino derivatives of avarone[9,10] also show interesting biological properties prompted us to prepare acyl, sulfide, and further amino derivatives of avarol and evaluate their biological properties.

Psoriasis is a chronic inflammatory skin disease mainly characterized by abnormal keratinocyte proliferation and accumulation of polymorphonuclear leukocytes in the skin.[11] Although the pathogenesis of psoriasis still remains unclear, there is growing evidence supporting the importance of reactive oxygen species (ROS) in the development of psoriatic lesions.[12,13] Uncontrolled production of active oxygen species leads to peroxidative damage to membranes of the skin, a tissue that is particularly vulnerable to the effects of this species.[14] In fact, increased reactive oxygen species and insufficient antioxidant activity have been determined in psoriatic lesions.[12]

Eicosanoids have also been suspected to play an important role in the pathophysiology of psoriasis. These metabolites affect blood vessels and inflammatory cells, contributing to dilate capillaries in the dermis and increase leukocyte infiltration and epidermal cell growth.[15] In this way, keratinocytes are able to enhance the rate of cell proliferation through increased PGE_2.[16,17]

Anthralin is among the most widely used drugs in the treatment of psoriasis. However, its clinical efficacy is limited by the side-effects of irritation and staining of the uninvolved skin.[18,19] The mechanism of anthralin-induced skin irritation is not completely understood, but several studies have suggested that it may be associated in part with the formation of active oxygen species or anthralin-derived radicals and the subsequent lipid peroxidation.[20−23] In the last years, the development of topically active compounds, which should obviate this drawback, has been highly desirable for the treatment of psoriatic lesions.[24]

On the basis of antioxidant properties of avarol and the role of ROS generation in the pathogenesis of psoriasis, we undertook the synthesis and possible structure−activity relationships for 14 avarol derivatives as potential antipsoriatic agents by inhibition of superoxide generation in activated human neutrophils or reduction of cell proliferation and PGE_2 generation in the cultured human keratinocyte HaCaT cell line. In addition, all the compounds have been tested on keratinocyte cell viability to discard any sign of cytotoxicity that could explain cell growth inhibition.

Results and Discussion

Avarol (**1**) was isolated from the sponge *Dysidea avara*,[25] collected in the Bay of Naples, Italy. Ester derivatives (**3**−**5**) of avarol were obtained by adding the corresponding acyl chloride to a solution of avarol in pyridine. The compound **6** was obtained by adding thiosalicylic acid to a solution of avarone (**2**), obtained by Ag_2O oxidation of avarol, in ethanol. Amino derivatives (**7**−**16**) were generally obtained by slowly adding the corresponding amine dissolved in basic solution to a dilute solution of avarone in ethanol or ethanol−water (1:1).[9]

For all the amines (methylamine, vanillylamine, tryptamine, benzylamine, and *p*-methoxybenzylamine) two isomers were obtained with substitution at 3′ (**7**, **9**, **11**, **13**, and **15**) and 4′ (**8**, **10**, **12**, **14**, and **16**) of the benzoquinone ring, as previously described[9] for other amino derivatives. The position of the substituent was determined by the analysis of ^{1}H NMR spectra. Signals of protons in the benzoquinone ring are doublets in 3′-substituted compounds and singlets in 4′-substituted compounds.

Initial radical-scavenging properties were obtained using 2,2-diphenyl-1-picrylhydrazyl (DPPH)[26,27] as a TLC spray

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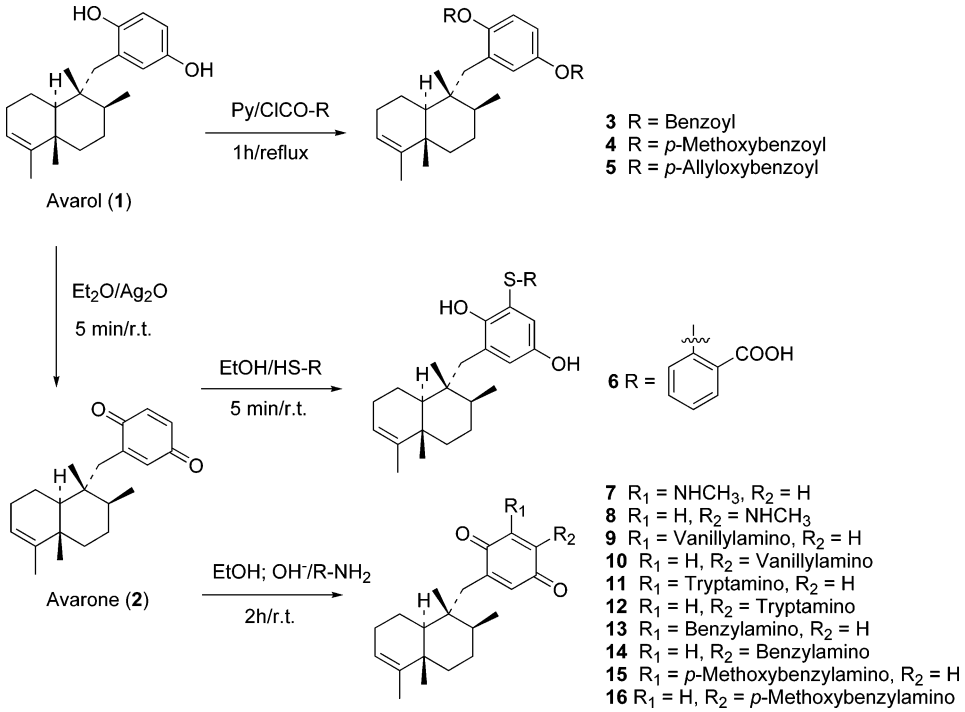
Scheme 1. Synthesis and Structure of Compounds **1**−**16**



reagent. Active compounds were further tested in solution. Avarol (**1**) showed the most potent antioxidant activity, with IC_{50} 18 μM, while **6**, **9**, and **10** exhibited moderate potencies, with IC_{50} 34, 95, and 98 μM, respectively.

In the assay of ROS generation in stimulated human neutrophils, all the compounds were tested at 5 μM. As expected, avarol (**1**) (IC_{50} 3.6 μM) and the reference compound fraxetin inhibited the generation of oxygen-derived species in a concentration-dependent manner. Compound **6** also potently reduced this parameter (IC_{50} 2.5 μM), while compounds **7** and **8** exerted only a weak inhibitory effect at 5 μM. Anthralin did not affect chemiluminescence at the concentration used. It has been reported that during the redox reaction, avarol/avarone undergoes a one-electron transfer with formation of a semiquinone free radical that acts as a radical scavenger.[5] The potent antioxidant properties showed by compound **6** could also be related to the presence of a hydroquinone moiety. We believe that the stability of acyl derivatives in the biological medium with the difficulty to produce free hydroquinone by hydrolysis is the main aspect of the loss of their activity in the tested bioassays. The stability of the quinone form of amino derivatives is at least in part responsible for the diminution of some aspect of biological activities, because they can be reduced in biological medium only with difficulty. On the other hand, it should be noted that none of the avarol derivatives caused cytotoxicity in the lactate dehydrogenase (LDH) assay.[28]

The human HaCaT keratinocyte cell line was used as a model for a highly proliferative epidermis useful in the evaluation of antipsoriatic agents.[29] All the compounds were tested for antiproliferative activity in comparison to the antipsoriatic drug anthralin. As shown in Table 1, compounds **7**, **8**, **10**−**12**, and **14** significantly reduced cell proliferation at 5 μM (Table 1). Compound **7** was the most potent, with an IC_{50} close to that of anthralin. Results showed that there were no obvious requirements for the more potent antiproliferative activity of compound **7** because only minor structural changes, such as variation of the position of the methylamino function in the quinone ring (compound **8**), decreased potency. To confirm that inhibition of keratinocyte growth was not a result of membrane damage, cell viability was assessed on the basis of leakage of LDH into the culture medium.[28] Treatment of HaCaT cells with standard anthralin caused remarkable damage to plasma membrane integrity (59% toxicity at 5 μM) in contrast to compound **7** and the rest of the avarol derivatives, which do not present any sign of cytotoxicity (Table 1).

As shown in Table 1, some avarol derivatives also significantly inhibited the generation of PGE_2, compound **6** being the most potent. The pronounced effect of compound **6** on PGE_2 levels could be related to the presence of a thiosalicylic function at the hydroquinone moiety, which could act through cyclo-oxygenase (COX) inhibition, in a manner similar to nonsteroidal anti-inflammatory drugs (NSAIDs).[30] As expected, NS-398, used as COX-2 inhibitor reference compound, potently inhibited PGE_2 levels in the culture cell.

Results obtained in the present study show that it is difficult to establish a clear relation between inhibition of keratinocyte cell growth and reduction of superoxide or PGE_2 generation. Although compound **6** was a potent inhibitor of ROS and PGE_2 production, it was not active as an inhibitor of HaCaT proliferation at 5 μM. In contrast, compound **7** was the most potent antiproliferative derivative but presented weak or negative effects on the other measured parameters. These results suggest a different mode of action for the compounds and also prove how difficult it is to find a possible structure−activity relationships. However, compounds **6** and **7** offer interesting perspectives because their profile and potency may have relevance in the inhibition of inflammatory response (compound **6**) and psoriatic pathologies (compound **7**). It is interesting to note that compound **7** retains the potent antiproliferative activity of the antipsoriatic anthralin but in contrast does not present any cell toxicity. Moreover, compound **7**, by exerting antioxidant properties, could also contribute to reduce the possible undesirable effects derived

Table 1. Superoxide Generation in Human Neutrophils: Cell Proliferation, PGE_2 Production, and Cytotoxicity in the HaCaT Keratinocyte Cell Line

	chemiluminescenc[a]		^{3}H-thymidine[b]		PGE_2[c]		LDH release[d]
	% I (5 μM)	IC_{50}	%I (5 μM)	IC_{50}	%I (5 μM)	IC_{50}	(A_{550nm} mU)
avarol	71.7 ± 4.7[f]	3.6 μM	18.9 ± 2.4		4.9 ± 3.3		151 ± 5
3	14.2 ± 4.7		19.6 ± 7.0		39.4 ± 8.5		152 ± 1
4	11.5 ± 2.2		12.5 ± 4.1		8.3 ± 6.2		155 ± 2
5	25.8 ± 5.6		36.3 ± 5.5		1.6 ± 1.6		158 ± 1
6	90.2 ± 4.4[f]	2.5 μM	22.6 ± 2.2		72.9 ± 6.5[f]	2.6 μM	159 ± 2
7	33.8 ± 2.7[e]		73.2 ± 2.9[f]	4.5 μM	14.9 ± 9.7		169 ± 9
8	37.8 ± 2.8[e]		33.5 ± 3.9[e]		54.8 ± 3.8[f]		157 ± 3
9	25.5 ± 2.9		16.6 ± 6.9		45.9 ± 9.1		153 ± 1
10	36.8 ± 5.2		35.0 ± 3.4[e]		38.9 ± 4.2		148 ± 2
11	25.2 ± 3.6		33.3 ± 3.3[e]		47.1 ± 5.3[e]		151 ± 1
12	26.7 ± 4.0		36.7 ± 4.6		50.4 ± 4.4[e]		150 ± 2
13	26.8 ± 1.1		25.6 ± 4.9		47.8 ± 5.1[e]		163 ± 3
14	17.6 ± 2.0		40.8 ± 3.8[f]		32.2 ± 5.1		158 ± 1
15	17.3 ± 1.9		24.5 ± 3.6		55.4 ± 4.0[f]		178 ± 1
16	10.8 ± 1.7		16.8 ± 4.8		13.1 ± 8.0		152 ± 1
anthralin	31.2 ± 6.5		73.8 ± 3.2[f]	2.9 μM	ND		285 ± 34[f]
fraxetin	76.4 ± 3.3[f]	1.0 μM	ND		ND		ND
NS-398	ND		ND		93.8 ± 5.3[f]	10 nM	ND
triton X	ND		ND		ND		376 ± 4[f]

[a] Results show percentages of inhibition (%I) at 5 μM. IC_{50} was determined for those compounds that reached 50% inhibition. Statistical evaluation included one-way analysis of variance followed by Dunnett's *t*-test for multiple comparisons. ND = not determined. Superoxide generation was measured by chemiluminescence with luminol. Fraxetin was used as antioxidant reference compound. [b] Results show percentages of inhibition (%I) at 5 μM. IC_{50} was determined for those compounds that reached 50% inhibition. Statistical evaluation included one-way analysis of variance followed by Dunnett's *t*-test for multiple comparisons. Antiproliferative activity was determined by the inhibition of ^{3}H-thymidine incorporation in HaCaT. [c] Results show percentages of inhibition (%I) at 5 μM. IC_{50} was determined for those compounds that reached 50% inhibition. Statistical evaluation included one-way analysis of variance followed by Dunnett's *t*-test for multiple comparisons. PGE_2 levels were determined by RIA. NS-398 was assayed as COX-2 reference inhibitor. [d] Cytotoxicity was determined by LDH release (mU) after 24 h treatment with 5 μM test compound. Data represent mean ± SEM (n = 6−12). [e] $P < 0.05$. [f] $P < 0.01$.

from respiratory burst of neutrophils infiltrated in the psoriatic skin.[13]

In conclusion, the present study suggests that the hydroquinone moiety and thiosalicylic function of compound **6** can offer interesting anti-inflammatory properties as antioxidant and inhibitor of PGE_2 release. In addition, the results obtained with compound **7** and its improved ratio of antiproliferative activity to cytotoxicity as compared to anthralin can be interesting as a possible antipsoriatic drug. These results confirm that pharmacological study of avarol and its derivatives can offer opportunities for discovering novel therapeutic agents.

Experimental Section

General Experimental Procedures. Melting points were determined using a Kofler hot-stage microscope and are uncorrected. Optical rotations were measured on a JASCO DIP 370 polarimeter, using a 10 cm microcell. UV spectra were obtained on a Varian DMS 90 spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded at 500 and 125 MHz, respectively, on a Bruker AMX-500 spectrometer in $CDCl_3$, using the residual $CDCl_3$ resonance at 7.26 and 77.0 ppm as internal references, respectively. Only chemical shifts of hydroquinone and acyl residues are reported because all other signals belonging to the sesquiterpenoid portion were earlier reported.[25,31] LRMS and HRMS were recorded on a JEOL JMS D-300 and an AEI MS-50, respectively. Column chromatography was carried out on Merck silica gel 60.

Materials. Avarol (**1**) was isolated from *Dysidea avara*,[25] which was collected in the Bay of Naples, Italy. Benzoyl chloride, *p*-methoxybenzoyl chloride, allyl bromide, *p*-hydroxybenzoic acid, thiosalicylic acid, methylamine, vanillylamine, tryptamine, benzylamine, and *p*-methoxybenzylamine were obtained from Sigma-Aldrich (Milano, Italy). *p*-Allyloxybenzoyl chloride was obtained by adding allyl bromide to a solution of *p*-hydroxybenzoic acid dissolved in 10% KOH−methanol, maintaining the solution at reflux for 1 h. Workup with aqueous 1 N HCl and extraction with Et_2O afforded *p*-allyloxybenzoic acid, mp 167−169 °C (from ethanol), which was converted to *p*-allyloxybenzoyl chloride with thionyl chloride at reflux in dimethylformamide. The rest of the reagents used in the biological tests were obtained from Sigma Chemicals (St. Louis, MO).

Synthesis of Dibenzoyl Avarol (3), Di-*p*-methoxybenzoyl Avarol (4), and Di-*p*-allyloxybenzoyl Avarol (5). Benzoyl chloride, *p*-methoxybenzoyl chloride, and *p*-allyloxybenzoyl chloride (150 mg each) were individually added to a solution of avarol (100 mg) in pyridine (2 mL) for 1 h at reflux. The excess solvent reagents were removed in vacuo, and the residues were partitioned between H_2O and Et_2O. The ether extracts were dried over anhydrous Na_2SO_4, and the solvent was evaporated to obtain an amorphous solid, which was purified on a silica gel column (*n*-hexane−Et_2O, 4:1). The esters were crystallized from EtOH to give compounds **3**−**5** (yield 100% for each one).

Dibenzoyl avarol (3): mp 158−159 °C; $[\alpha]^{25}_D$ +7.4° (*c* 0.13, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 234 (4.13), 228 (4.11); ^{1}H NMR ($CDCl_3$) δ 8.23 (4H, d, J = 7.8 Hz, H2″ and H6″), 7.66 (2H, m, H4″), 7.53 (4H, m, H3″ and H5″), 7.14 (3H, m, H3′, H4′, and H6′); EIMS *m*/*z* 522 $[M]^+$ (2), 507 (1), 417 (5), 332 (30), 191 (100), 105 (95); HREIMS *m*/*z* 522.2773 (calcd for $C_{35}H_{38}O_4$, 522.2770).

Di-*p*-methoxybenzoyl avarol (4): mp 175−177 °C; $[\alpha]^{25}_D$ +10.2° (*c* 0.11, $CHCl_3$); UV (MeOH) λ_{max} (logϵ) 262 (4.29), 219 (4.07); ^{1}H NMR ($CDCl_3$) δ 8.16 (4H, d, J = 8.8 Hz, H2″ and H6″), 7.11 (3H, m, H3′, H4′, and H6′), 6.99 (4H, d, J = 8.8 Hz, H3″ and H5″); EIMS *m*/*z* 582 $[M]^+$ (0.1), 447 (3), 392 (15), 191 (90), 135 (100); HREIMS *m*/*z* 582.2977 (calcd for $C_{37}H_{42}O_6$, 582.2981).

Di-*p*-allyloxybenzoyl avarol (5): mp 149−150 °C; $[\alpha]^{25}_D$ +10.1° (*c* 0.11, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 265 (4.77), 215 (4.25); ^{1}H NMR ($CDCl_3$) δ 8.15 (4H, d, J = 8.8 Hz, H2″ and H6″), 7.10 (3H, m, H3′, H4′, and H6′), 7.00 (4H, d, J = 8.8 Hz, H3″ and H5″), 6.08 (2H, m, H2‴), 5.45 (2H, d, J = 17.3 Hz, H3a‴), 5.34 (2H, d, J = 10.5 Hz, H3b‴), 4.64 (4H, d, J = 5.2 Hz, H1‴); EIMS *m*/*z* 634 $[M]^+$ (0.3), 594 (0.3), 473 (1), 444 (15), 191 (90), 161 (100); HREIMS *m*/*z* 634.3300 (calcd for $C_{41}H_{46}O_6$, 634.3294).

Synthesis of Avarol-3′-thiosalicylate (6). Thiosalicylic acid (100 mg) dissolved in EtOH (10 mL) was added to a solution of avarone (**2**) (100 mg) in EtOH (20 mL) and stirred for 5 min at room temperature. After evaporation of EtOH, the residue was chromatographed on a Si gel column and eluted with petroleum ether−Et_2O−HOAc (7:3:0.1) to give avarol-3′-thiosalicylate (**6**) (90 mg): mp 113−115 °C ($CHCl_3$−MeOH); $[\alpha]^{25}_D$ −0.33° (*c* 0.014, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 317 (4.07); ^{1}H NMR ($CDCl_3$) δ 6.91 (H-6′, d, J = 2.5 Hz), 6.79 (H-4′, d, J = 2.5 Hz); EIMS *m*/*z* 468 $[M + 2]^+$ (0.4), 466 $[M]^+$ (10), 276 (18), 258 (22), 189 (30), 135 (30), 107 (35), 95 (100); HREIMS *m*/*z* 466.2183 (calcd for $C_{28}H_{34}O_4S$, 466.2178).

Synthesis of 7 and 8. Avarone (**2**) (200 mg) was treated with $MeNH_2$−HCl as previously described,[9] and 42 mg of **7** and 73 mg of **8** were recovered. Compounds **7** and **8** were identified by comparison with authentic samples.

Synthesis of 9 and 10. Vanillylamine (200 mg) was dissolved in a saturated solution of $NaHCO_3$ (30 mL), added to a solution of avarone (**2**) (100 mg) in EtOH (30 mL), and stirred for 24 h at room temperature. After evaporation of EtOH, the remaining aqueous solution was extracted with $CHCl_3$, and the extract was chromatographed on a Si gel column and eluted with petroleum ether−Et_2O (7:3). The more polar component was 3′-vanillylaminoavarone (**9**) (30 mg): mp 145−146 °C ($CHCl_3$−MeOH); $[\alpha]^{25}_D$ −72.4° (*c* 0.01, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 283 (3.82), 486 (3.18); ^{1}H NMR ($CDCl_3$) δ 6.38 (H-6′, d, J = 2.3 Hz), 5.50 (H-4′, d, J = 2.3 Hz); EIMS *m*/*z* 463 $[M]^+$ (1.5), 448 (3), 312 (2), 273 (30), 191 (20), 175 (20), 139 (90) 137 (100); HREIMS *m*/*z* 463.2725 (calcd for $C_{29}H_{37}NO_4$, 463.2722). The less polar component was 4′-vanillylaminoavarone (**10**) (36 mg): mp 159−161 °C ($CHCl_3$−MeOH); $[\alpha]^{25}_D$ −50.4° (*c* 0.01, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 284 (4.14), 486 (3.33); ^{1}H NMR ($CDCl_3$) δ 6.38 (H-6′, s), 5.52 (H-3′, s); EIMS *m*/*z* 463 $[M]^+$ (5), 448 (6), 312 (18), 274 (90), 273 (85) 189 (60), 175 (50), 139 (90) 137 (100); HREIMS *m*/*z* 463.2719 (calcd for $C_{29}H_{37}NO_4$, 463.2722).

Synthesis of 11 and 12. Tryptamine (200 mg) dissolved in a saturated solution of $NaHCO_3$ (30 mL) was added to a solution of avarone (**2**) (100 mg) in EtOH (30 mL). After the usual workup, the $CHCl_3$ extract was chromatographed on a Si gel column and eluted with petroleum ether−Et_2O (6:4) to give 3′-tryptaminoavarone (**11**) as the more polar component (32 mg): mp 183−184 °C ($CHCl_3$−MeOH); $[\alpha]^{25}_D$ −55.0° (*c* 0.015, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 283 (4.17), 493 (3.40); ^{1}H NMR ($CDCl_3$) δ 6.26 (H-6′, d, J = 2.3 Hz), 5.37 (H-4′, d, J = 2.3 Hz); EIMS *m*/*z* 470 $[M]^+$ (1.5), 455 (2), 341 (8), 282 (12), 151 (65), 130 (100); HREIMS *m*/*z* 470.2937 (calcd for $C_{31}H_{38}N_2O_2$, 470.2933), and 4′-tryptaminoavarone (**12**) as the less polar component (18 mg): mp 163−164 °C ($CHCl_3$−MeOH); $[\alpha]^{25}_D$ −33.5° (*c* 0.01, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 282 (4.41), 496 (3.84); ^{1}H NMR ($CDCl_3$) δ 6.23 (H-6′, s), 5.38 (H-3′, s); EIMS *m*/*z* 470 $[M]^+$ (8), 455 (12), 340 (18), 281 (90), 189 (10), 175 (50), 150 (90) 144 (900), 130 (100); HREIMS *m*/*z* 470.2935 (calcd for $C_{31}H_{38}N_2O_2$, 470.2933).

Synthesis of 13 and 14. Benzylamine (2 mL) dissolved in a saturated solution of $NaHCO_3$ (30 mL) was added to a solution of avarone (**2**) (100 mg) in EtOH (30 mL). After the usual workup, the $CHCl_3$ extract was chromatographed on a Si gel column and eluted with petroleum ether−Et_2O (8:2) to give 3′-benzylaminoavarone (**13**) as the more polar component (33 mg): mp 82−83 °C (hexane); $[\alpha]^{25}_D$ −90.3° (*c* 0.015, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 288 (3.82), 486 (3.44); ^{1}H NMR ($CDCl_3$) δ 6.38 (H-6′, d, J = 2.3 Hz), 5.48 (H-4′, d, J = 2.3 Hz); EIMS *m*/*z* 417 $[M]^+$ (3), 402 (30), 227 (100), 173 (75), 150 (80), 138 (95); HREIMS *m*/*z* 417.2663 (calcd for $C_{28}H_{35}NO_2$, 417.2668), and 4′-benzylaminoavarone (**14**) as the less polar component (46 mg): mp 107−108 °C (hexane); $[\alpha]^{25}_D$ −44.0° (*c* 0.02, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 290 (4.10), 482 (3.51); ^{1}H NMR ($CDCl_3$) δ 6.38 (H-6′, s), 5.48 (H-3′, s); EIMS *m*/*z* 417 $[M]^+$ (5), 402 (16), 227 (100), 175 (20), 149 (15), 138 (10); HREIMS *m*/*z* 417.2670 (calcd for $C_{28}H_{35}NO_2$, 417.2668).

Synthesis of 15 and 16. 4-Methoxybenzylamine (1.8 mL) dissolved in a saturated solution of $NaHCO_3$ (30 mL) was added to a solution of avarone (**2**) (100 mg) in EtOH (30 mL). After the usual workup, the $CHCl_3$ extract was chromatographed on a Si gel column and eluted with petroleum ether−Et_2O (8:2) to give 3′-*p*-methoxybenzylaminoavarone (**15**) as the more polar component (28 mg): mp 90−91 °C (hexane); $[\alpha]^{25}_D$ −58.2° (*c* 0.009, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 283 (3.79), 485 (3.38); ^{1}H NMR ($CDCl_3$) δ 6.37 (H-6′, d, J = 2.3 Hz), 5.48 (H-4′, d, J = 2.3 Hz); EIMS *m*/*z* 447 $[M]^+$ (10), 432 (30), 257 (95), 173 (75), 160 (30), 138 (80), 121 (100); HREIMS *m*/*z* 447.2770 (calcd for $C_{29}H_{37}NO_3$, 447.2773), and 4′-*p*-methoxybenzylaminoavarone (**16**) as the less polar component (38 mg): mp 131−132 °C (hexane); $[\alpha]^{25}_D$ −80.1° (*c* 0.01, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 284 (4.10), 486 (3.43); ^{1}H NMR ($CDCl_3$) δ 6.37 (H-6′, s), 5.51 (H-3′, s); EIMS *m*/*z* 447 $[M]^+$ (10), 432 (15), 257 (100), 175 (55), 160 (80), 138 (70), 121 (95); HREIMS *m*/*z* 447.2777 (calcd for $C_{29}H_{37}NO_3$, 447.2773).

Biological Tests. Free-Radical Scavenging Activity. TLC Autographic Assay. A 5 μg sample of compounds **1** and **3**−**16** was applied on a TLC plate. After developing [eluent: light petroleum−Et_2O (1:1)] and drying, TLC plates were sprayed with a 0.2% DPPH solution in MeOH. The plates were examined 30 min after spraying. Active compounds (**1**, **6**, **9**, and **10**) appear as yellow spots against a purple background.

Assay in Solution. Solutions of compounds **1**, **6**, **9**, and **10** in MeOH, at different concentrations, were prepared and adjusted to 2 mL total volume with 0.7 mL of DPPH−MeOH solution (6 mg/50 mL; 0.1 mM final concentration). The absorbance at 517 nm was determined after 30 min, and the percent free radical inhibition was calculated and plotted to obtain the IC_{50} value. The IC_{50} value denotes the concentration of compound required to scavenge 50% DPPH free radical.

ROS Generation in Human Neutrophils. Human neutrophils were obtained from citrated blood of healthy volunteers and purified as previously described.[32] Neutrophils (2.5×10^6 cells/mL) were incubated with luminol (40 μM) and stimulated with 1 μM 12-O-tetradecanoyl phorbol 13-acetate (TPA) for 7 min. ROS generation was determined as chemiluminescence recorded in a Microbeta trilux counter (Wallac, Turku, Finland).

Cytotoxicity Assay. The cytotoxicity of products was assessed by determination of lactate dehydrogenase (LDH) release in supernatants obtained after treatment of neutrophils and keratinocytes with tested compounds.[28]

Antiproliferative Assay. The human keratinocyte cell line HaCaT was provided by Dr. N. E. Fusenig (Heidelberg, Germany). The cell line was cultured in DMEM medium with 10% fetal calf serum (FCS), 100 U/mL penicillin, and 100 μg/mL streptomycin, in a humidified incubator (5% CO_2 at 37 °C.) Keratinocytes (4×10^4/200 μL/well) were grown during 24 h on 96-well multidishes. The medium was replaced, and test compounds or vehicle (1% EtOH) was added. After 24 h of incubation, cells were pulsed for 6 h with methyl-^{3}[H]-thymidine (1 μCi/mL). Cells were washed and thymidine incorporation was measured with a Microbeta trilux counter after addition of 200 μL of liquid scintillation counting. All the compounds were tested for antiproliferative activity in comparison to the antipsoriatic drug anthralin.

PGE_2 Generation Assay. HaCaT cells (1×10^4/200μL/well) were seeded in 96-well plates and cultivated for 24 h. After being washed, test compounds (5 μM) and arachidonic acid (10 μM) were added for 24 h incubation. Finally, supernatants were collected for the determination of PGE_2 by radioimmunoassay.[33]

Statistical Analysis. The results are presented as mean ± SEM. Inhibitory concentration 50% (IC_{50}) values were calculated from at least four significant concentrations (n = 6). The level of statistical significance was determined by analysis of variance (ANOVA), followed by Dunnett's *t*-test for multiple comparisons.

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References and Notes

(1) De Rosa, S. In *Natural products in the new millennium: Prospects and industrial application*; Rauter, A. P., Palm, F. B., Justino, J., Araujo, M. E., dos Santos, S. P., Eds.; Kluwer Academic Publishers: The Netherlands, 2002; pp 441−461.
(2) Belisario, M. A.; Maturo, M.; Avagnale, G.; De Rosa, S.; Scopacasa, F.; De Caterina, M. *Pharmacol. Toxicol.* **1996**, *79*, 300−304.
(3) Shen, Y. C.; Lu, C. H.; Chakraborty, R.; Kuo, Y. H. *Nat. Prod. Res.* **2003**, *17*, 83−89.
(4) Ferrándiz, M. L.; Sanz, M. J.; Bustos, G.; Payá, M.; Alcaraz, M. J.; De Rosa, S. *Eur. J. Pharmacol.* **1994**, *253*, 75−82.
(5) Belisario, M. A.; Maturo, M.; Pecce, R.; De Rosa, S.; Villani, G. R. *Toxicology* **1992**, *72*, 221−233.
(6) De Rosa, S.; De Giulio, A.; Strazzullo, G. *Trends Org. Chem*. **1991**, *2*, 127−141.
(7) Belisario, M. A.; Pecce, R.; Maturo, M.; De Rosa, S. *Toxicology* **1994**, *86*, 89−108.
(8) Crispino, A.; De Giulio, A.; De Rosa, S.; Strazzullo, G. *J. Nat. Prod.* **1989**, *52*, 646−648.
(9) Cozzolino, R.; De Giulio, A.; De Rosa, S.; Strazzullo, G. *J. Nat. Prod.* **1990**, *53*, 699−702.
(10) De Giulio, A.; De Rosa, S.; Stazzullo, G.; Diliberto, L.; Obino, P.; Marongiu, M. E.; Pani, A.; La Colla, P. *Antivir. Chem. Chemother.* **1991**, *2*, 223−227.
(11) Rogalski, C.; Meyer-Hoffert, U.; Proksch, E.; Wiedow, O. *J. Invest. Dermatol.* **2002**, *118*, 49−54.
(12) Yildirim, M.; Inaloz, H. S.; Baysal, V.; Delibas, N. *J. Eur. Acad. Dermatol. Venereol.* **2003**, *17*, 34−36.
(13) Utas, S.; Kose, K.; Yazici, C.; Akdas, A.; Kelestimur, F. *Clin. Biochem.* **2002**, *35*, 241−246.
(14) Trenam, C. W.; Blake, D. R.; Morris, C. J. *J. Invest. Dermatol.* **1992**, *99*, 675−682.
(15) Ikai, K. *J. Dermatol. Sci.* **1999**, *21*, 135−146.
(16) Rys-Sikora, K. E.; Konger, R. L.; Schoggins, J. W.; Malaviya, R.; Pentland, A. P. *Am. J. Physiol. Cell Physiol.* **2000**, *278C*, 822−833.
(17) Pentland, A. P.; Needleman, P. *J. Clin. Invest.* **1986**, *77*, 246−251.
(18) Parslew, R.; Friedmann, P. S. *Br. J. Dermatol.* **1999**, *141*, 469−474.
(19) Kemeny, L.; Ruzicka, T.; Braun-Falco, O. *Skin Pharmacol.* **1990**, *3*, 1−20.
(20) Lange, R. W.; Hayden, P. J.; Chignell, C. F.; Luster, M. I. *Inflamm. Res.* **1998**, *47*, 174−181.
(21) Muller, K.; Reindl, H.; Breu, K. *J. Med. Chem.* **2001**, *44*, 814−821.
(22) Muller, K. *Gen. Pharmacol.* **1996**, *27*, 1325−1335.
(23) Peus, D.; Beyerle, A.; Rittner, H. L.; Pott, M.; Meves, A.; Weyand, C.; Pittelkow, M. R. *J. Invest. Dermatol.* **2000**, *114*, 688−692.
(24) Muller, K. *Curr. Pharm. Des.* **2000**, *6*, 901−918.
(25) Minale, L.; Riccio, R.; Sodano, G. *Tetrahedron Lett.* **1974**, *38*, 3401−3404.
(26) Takao, T.; Kitatani, F.; Watanabe, N.; Yagi, A.; Sakata, K. *Biosci. Biotechnol. Biochem.* **1994**, *51*, 1780−1783.
(27) Cuendet, M.; Hostettmann, K.; Potterat, O.; Dyatmiko, W. *Helv. Chim. Acta* **1997**, *80*, 1144−1152.
(28) Babson, A. L.; Phillips, G. E. *Clin. Chim. Acta* **1965**, *12*, 210−215.
(29) Boukamp, P.; Petrussevska, R. T.; Breitkreutz, D.; Hornung, J.; Markham, A.; Fusenig, N. E. *J. Cell Biol.* **1988**, *106*, 761−771.
(30) Fitzgerald, G. A. *Nat. Rev. Drug Discovery* **2003**, *2*, 879−890.
(31) De Rosa, S.; Minale, L.; Riccio, R.; Sodano, G. *J. Chem. Soc., Perkin Trans. 1* **1976**, 1408−1414.
(32) Bustos, G.; Ferrándiz, M. L.; Sanz, M. J.; Payá, M.; Alcaraz, M. J. *Naunyn Schmiedebergs Arch. Pharmacol.* **1995**, *351*, 298−304.
(33) Moroney, M. A.; Alcaraz, M. J.; Forder, R. A.; Carey, F.; Hoult, J. R. *J. Pharm. Pharmacol.* **1988**, *40*, 787−792.

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