Expedited Articles

Novel Antiproliferative Agents Derived from Lavendustin A

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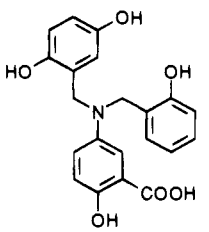
The active partial structure of the potent tyrosine kinase inhibitor lavendustin A was derivatized in the search for novel agents against cellular proliferation. The antiproliferative potential of the new derivatives was determined using the human keratinocyte cell line HaCaT as the primary test system. Whereas the lavendustin A partial structure is ineffective in inhibiting cell proliferation, esterification of its carboxylic acid function leads to measurable antiproliferative activity. Additional O-methylation of the 2,5-dihydroxyphenyl moiety yields activity in the micromolar range. Further substantial increases in activity are achieved by replacing the nitrogen with oxygen and carbon within the 2,5-dimethoxyphenyl series (but not within the 2,5-dihydroxyphenyl analogs) leading to 5-[2-(2,5-dimethoxyphenyl)ethyl]-2-hydroxybenzoic acid methyl ester (**13**) as the most potent analog identified to date. These increases in antiproliferative activity are paralleled, however, by the disappearance of activity against the epidermal growth factor receptor-associated tyrosine kinase, suggesting another mechanism of action.

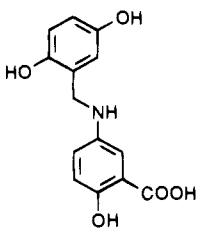
Introduction

Activation of protein tyrosine kinases is one of the main mechanisms by which external stimuli are transmitted into cells. Their regulated activity plays an important role in normal developmental and regenerative processes. Overexpression of (or mutation in) protein tyrosine kinases can result in the loss of growth control leading to cancer and other disorders of cell proliferation.[1] For example, the epidermal growth factor receptor (EGF-R), which is endowed with tyrosine kinase activity, and its endogenous ligand transforming growth factor α (TGFα) are overexpressed in several tumors, as well as in hyperproliferating keratinocytes of psoriatic lesions.[2-5] Therefore, tyrosine kinase inibitors might be useful agents for certain hyperproliferative disorders due to selectivity against abnormal cell proliferation.

Recently, the novel microbial secondary metabolite lavendustin A (**1**) and its partial structure **2** were reported to potently inhibit the EGF-R tyrosine kinase in A431 cell-free extracts but not in intact A431 cells.[6] The lack of cellular activity was attributed to poor cell penetration.[7] On the basis of the lavendustin A partial structure **2**, we initiated a medicinal chemistry program with the aim of improving cell penetration and antiproliferative activity. This led to the discovery of novel structures, which exhibit high antiproliferative activity against tumor and keratinocyte cell lines but are inactive against the EGF-R tyrosine kinase.



Lavendustin A (1)



Partial Structure **2**

Chemistry

Benzylamine compounds **2–5** were synthesized by condensation of 2,5-dihydroxy- or 2,5-dimethoxybenzaldehyde with 5-aminosalicylic acid or its methyl ester and reduction of the Schiff bases with sodium cyanoborohydride. Analog **6** was obtained using the same procedure starting from 2,5-dimethoxyaniline and 5-formylsalicylic acid methyl ester. Benzyl ether compound **8** was prepared by selective O-alkylation at the 5-hydroxy function of 2,5-dihydroxybenzoic acid methyl ester with 2,5-dimethoxybenzyl bromide using 1 equiv of potassium carbonate in acetone. Alkaline hydrolysis afforded the free acid **7**. Reaction of 2-acetoxy-5-(bromomethyl)benzoic acid methyl ester with sodium 2,5-dimethoxyphenolate in dimethylformamide and deprotection of the acetoxy group led to compound **9**. Stilbene derivatives **10** and **11** were synthesized by Wittig reaction from (2,5-dimethoxybenzyl)triphenylphosphonium bromide and 5-formylsalicylic acid methyl ester and separated by silica gel chromatography. Unprotected 5-formylsalicylic acid methyl ester could be used successfully only when lithium diisopropylamide (LDA) in tetrahydrofuran, prepared from a highly concentrated solution of *n*-butyllithium (10 M solution in hexane; Aldrich Chemical Co.), was used as base to

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deprotonate the phosphonium salt in the Wittig reaction. The use of other bases or a higher percentage of hexane in the solvent mixture (from the preparation of LDA using less concentrated solutions of *n*-butyllithium) gave substantially lower yields of products, probably due to insufficient solubility of the phenolate intermediate. Therefore, previous protection of the salicylic hydroxy group in the starting material was required in these reactions. Catalytic hydrogenation (palladium on charcoal, atmospheric pressure) of the stilbenes produced the reduced analog **13**, which was converted into the free acid **12** by alkaline hydrolysis. The dihydroxy compounds **14** and **15** were obtained from compounds **12** and **13** by dealkylation with boron tribromide. For purification of the salicylic acid analog **14**, treatment with aqueous glycerol was necessary in order to destroy residual boron complexes.

Results and Discussion of Biological Activities

The microbial metabolite lavendustin A (**1**) and its synthetic partial structure **2** (which was shown to be the active pharmacophore of **1**) potently inhibit the EGF-R tyrosine kinase in A431 cell-free extracts.[6] Both compounds feature a salicylic acid and a hydroquinone structural element. The lack of cellular activity of **1** was attributed to insufficient cell penetration caused by the polar functional groups, in particular the carboxylic moiety.[7] Therefore, several research groups initiated medicinal chemistry programs with **2** as the lead structure aimed at improving cell penetration and, hence, cellular activity. Whereas other researchers used tyrosine kinase assays for primary screening,[8-11] we used a cellular test system for lead optimization evaluating our derivatives primarily for their antiproliferative activity against the human keratinocyte cell line HaCaT.

As shown in Table 1, lack of cellular activity of compounds **1** and **2** was confirmed in the HaCaT keratinocyte proliferation assay. Methyl ester **3** demonstrated antiproliferative activity with an IC_{50} of 29 μM against HaCaT keratinocytes, supporting the hypothesis that cellular inactivity of **1** and **2** was due to insufficient cell penetration. O-methylation of the hydroquinone structural element of **2** produced the dimethoxy analog **4**, which was devoid of substantial antiproliferative activity. Additional esterification of the free carboxylic group in **4**, however, resulted in a marked increase of potency in the HaCaT keratinocyte proliferation assay. Thus, compound **5**, formally derived from the lead structure **2** by dimethylation plus esterification, exhibited antiproliferative activity in the low micromolar range with an IC_{50} of 1.5 μM against HaCaT keratinocytes.

In the following compound series, the spacer connecting the two aromatic ring systems was modified. Compound **6** (interchanged amino and methylene groups with respect to **5**) showed weak antiproliferative activity. Replacement of the nitrogen in structures **4** and **5** by oxygen led to the corresponding ether derivatives **7** and **8**. The acid compound **7** showed only weak activity (IC_{50} = 18 μM) but was significantly superior to the aminosalicylic acids **1**, **2**, and **4**. The corresponding

Scheme 1

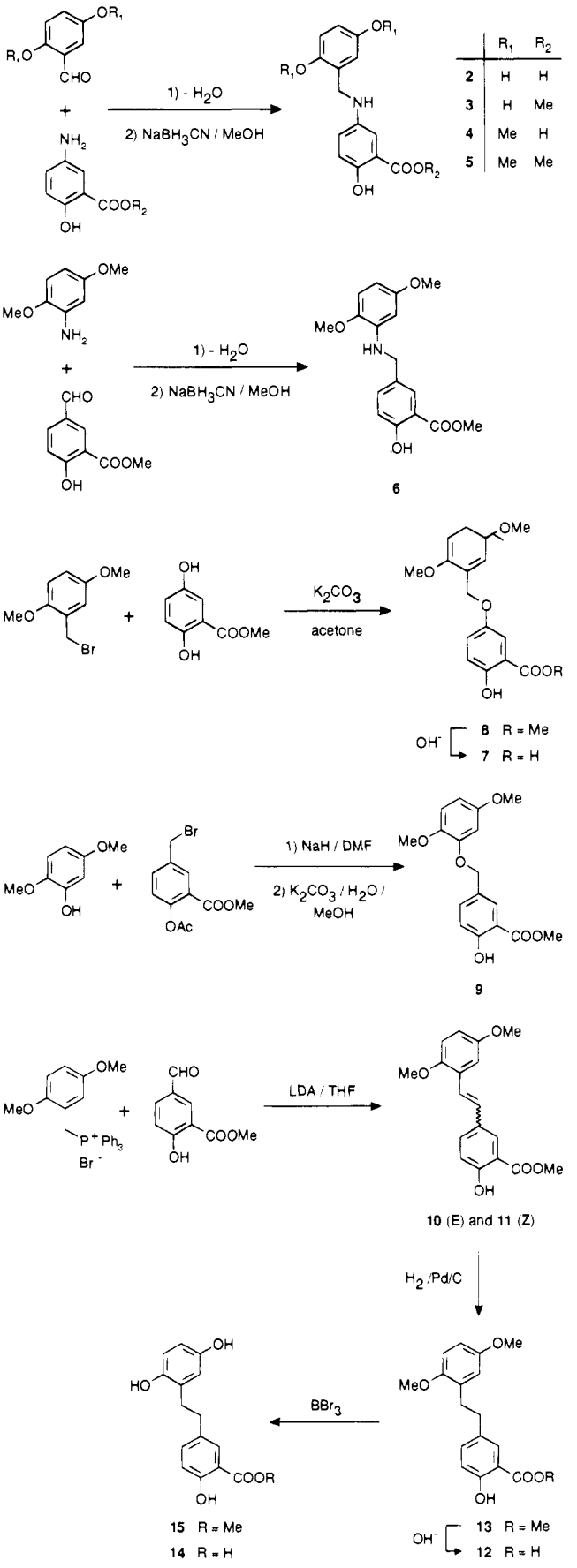

Table 1. Antiproliferative Activity of Compounds 1–15 on the HaCaT Keratinocyte Cell Line and Inhibition of EGF-R Tyrosine Kinase (TK) Activity by Selected Compounds

	IC_{50} (μM)	
no.	HaCaT	EGF-R TK
1	>100	4.1
2	≥100	2.9
3	29	24.4
4	92	>100
5	1.5	–
6	72	–
7	18	>100
8	0.31	>100
9	14	–
10	5.6	–
11	3.9	–
12	4.8	–
13	0.04	>100
14	>100	3.3
15	18	–

methyl ester **8** showed increased activity in the keratinocyte proliferation assay in comparison with all previously tested compounds and was the first compound with an IC_{50} below 1 μM. With factors of about 5 (between **7** and **4** and between **8** and **5**), the increase in antiproliferative activity (ether relative to amino series) was similar for both pairs of compounds. As already observed for compound **6**, reversing of the $-CH_2O-$ spacer between the two aromatic rings of **8** resulted in substantial reduction of activity (**9**).

Replacement of the amino function in structures **4** and **5** by a $-CH_2-$ group produced compounds **12** and **13**. With an IC_{50} of 4.8 μM, the acid derivative **12** was clearly superior to its amino (**4**) and ether (**7**) analogs. This improvement of activity was also observed for the corresponding methyl ester derivative: Compound **13** potently inhibited keratinocyte proliferation, showing an IC_{50} of 40 nM. Thus, the antiproliferative activity of compound **3**, which was the first structure exhibiting cellular activity, was enhanced by almost 3 orders of magnitude. In contrast, the stilbenes **10** and **11** were only moderately active in the HaCaT keratinocyte assay (Table 1). These findings suggested that a high degree of flexibility with regard to the relative spatial positions of the two aromatic ring systems might be important for high antiproliferative activity. Higher flexibility might also be the reason for the superior activity of the (2,5-dimethoxyphenyl)ethyl (**12**, **13**) and (2,5-dimethoxybenzyl)oxy (**7**, **8**) versus (2,5-dimethoxybenzyl)amino (**4**, **5**) analogs. In the benzylamino compounds, a preferred conformational arrangement of the molecule could be expected due to potential interaction of the amino function with the 2-methoxy group. In order to study whether introduction of the $-CH_2CH_2-$ spacer is also beneficial for activity of hydroquinone structures, the dihydroxy analogs **14** and **15** were synthesized. The acid derivative **14** had no significant effect on keratinocyte proliferation (as the amino analog **2**), and the methyl ester **15** showed only similar weak activity as the corresponding amino analog 3. Thus, the marked improvement of activity observed for the (2,5-dimethoxyphenyl)ethyl compounds (**12**, **13**) relative to their (2,5-dimethoxybenzyl)amino analogs (**4**, **5**) was not found within the dihydroxy series.

Selected compounds were tested for their inhibitory potential against the EGF-R tyrosine kinase under cell-free conditions (Table 1, third column). Lavendustin A inhibited the EGF-R tyrosine kinase activity completely and concentration-dependently with an IC_{50} of 4.1 μM. This value was not as low as that originally claimed[6] but agreed well with the value reported by other groups[9] and might reflect differences in assay conditions. The lavendustin A partial structure **2** showed similar inhibitory potency, confirming that it represents the active pharmacophore[6] of the natural product. The corresponding methyl ester **3**, while demonstrating cellular antiproliferative activity, was less active than **2** in inhibiting tyrosine kinase activity (Table 1). Interestingly, the IC_{50} values of **3** obtained in the enzyme assay and the keratinocyte proliferation assay are very similar (24.4 versus 29 μM). Surprisingly, analogs **4**, **7**, **8**, and **13** were found to be inactive against the EGF-R tyrosine kinase up to concentrations of 100 μM. These results contrasted in particular with the high antiproliferative potency observed for compounds **8** and **13** (Table 1). Since an aminosalicylic acid derivative (analog **4**) was within the series of enzymatically inactive analogs, it was not likely that structural modification of the spacer between the two aromatic ring systems (as in analogs **7**, **8**, and **13**) was responsible for loss of activity. It was striking, however, that these compounds collectively featured a 2,5-dimethoxyphenyl moiety instead of the hydroquinone residue present in the active compounds **1-3**. Therefore, we assumed that within lavendustin derivatives the hydroquinone structural element is essential for efficient inhibition of EGF-R tyrosine kinase. This is in full agreement with a recently reported SAR of tyrosine kinase inhibiting lavendustin derivatives[10] and was confirmed by the result obtained with the dihydroxyphenyl structure **14**. Compound **14** showed similar activity as lavendustin A (**1**) and its partial structure **2**. This agrees with the previously reported finding[9] that the amino function in lavendustin derivatives is not mandatory for potent inhibition of tyrosine kinase activity.

The negative results obtained with the 2,5-dimethoxyphenyl analogs in the tyrosine kinase assay suggested a different mode of action for the strong antiproliferative activity of compounds **8** and **13**. Extensive studies (to be reported in detail elsewhere) revealed that these compounds act by blocking the cell cycle at mitosis by perturbing the microtubules of the mitotic spindle apparatus.[12]

The most potent compounds in the keratinocyte proliferation assay (**8**, **13**) were additionally tested against tumor cell lines. Compound **8** inhibited the proliferation of the SK-BR-3 and MDA-231 cell lines with IC_{50} values (0.26–0.28 μM) very similar to those obtained in the keratinocyte assay (0.31 μM, Table 1). The antiproliferative potential of **13** against tumor cell lines was studied in more detail[13,14] in comparison with colchicine as reference compound. The activities against a panel of human and murine tumor cell lines are listed in Table 2. In contrast to colchicine, which inhibited the growth of all tumor cells at low nanomolar concentrations, compound **13** showed a specific high activity against mammary tumor cells. Thus, the proliferation of three out of four mammary tumor cell lines (MCF-7, SK-BR-3, MDA-MB-468) was inhibited by **13** with IC_{50} values ranging between 20 and 50 nM as compared to 150–500 nM for the other cell lines. The potent antiproliferative activity of compound **13** was confirmed

Table 2. Antiproliferative Effects of Compound **13** on a Panel of Tumor Cell Lines in Comparison with the Standard Colchicine[a]

cell name	IC_{50} (nM)[b]	
	13	colchicine
MCF-7	19.0 ± 8.5	15.3 ± 12.2
SK-BR-3	31.0 ± 1.4	5.0 ± 1.4
MDA-MB-468	46.5 ± 14.1	6.9 ± 0.9
A431	213 ± 28	14.0
LOVO	185.0 ± 7.1	6.0 ± 0.8
KB	191.5 ± 12.0	5.6 ± 1.6
K562	217.5 ± 31.8	14.1 ± 4.1
B16F1	269	nt[c]
U937	320	5.2
P388	337.5 ± 123.7	9.5 ± 3.5
SCL-1	383	nt[c]
L1210	415.0 ± 7.1	14.7 ± 11.0
MDA-MB-231	427.5 ± 130.8	20.5 ± 0.7
HELA	470.0 ± 212.1	18.0 ± 2.8

[a] Table taken from ref 14. [b] Results represent IC_{50} values of single experiments or the mean ± standard deviation of two independent experiments run in triplicate. [c] nt = not tested.

in vivo: it was found to be effective in a model of epidermal hyperplasia after topical application[13] and in selected tumor models after oral and intravenous application.[13,14]

In summary, etherification plus esterification of lavendustin A partial structures resulted in the identification of novel potent antiproliferative compounds. The most active analog so far, 5-[2-(2,5-dimethoxyphenyl)-ethyl]-2-hydroxybenzoic acid methyl ester (**13**), shows potent and selective antiproliferative activity *in vitro* against the human keratinocyte cell line HaCaT and several tumor cell lines. Furthermore, activity in several *in vivo* models of hyperproliferation indicates therapeutic potential against hyperproliferative skin diseases and cancer.

Experimental Section

Chemistry. Materials and Methods. 2,5-Dihydroxybenzaldehyde, 2,5-dimethoxybenzaldehyde, and 2,5-dimethoxyaniline were purchased from Fluka Chemie AG. 5-Aminosalicylic acid, 2,5-dihydroxybenzoic acid, and 5-formylsalicylic acid were purchased from Aldrich Chemical Co. and converted into the corresponding methyl esters by treatment with sulfuric acid in methanol. 2,5-Dimethoxybenzyl bromide,[15] 2,5-dimethoxyphenol,[16] and 2-acetoxy-5-(bromomethyl)benzoic acid methyl ester[17] were prepared according to published procedures. (2,5-Dimethoxybenzyl)triphenylphosphonium bromide (mp 217 °C) was synthesized from 2,5-dimethoxybenzyl bromide and triphenylphosphine in toluene.

Melting points were determined on a Reichert Thermovar microscope and are not corrected. The temperature is given in Celsius units. Thin-layer chromatography was performed using silica gel F_{254} plates (Merck) visualizing with UV or potassium permanganate. Column chromatography was performed using silica gel 60 (0.040–0.063 mm; Merck), pressure 3–5 bar. ^1H NMR spectra were recorded at 250 MHz (Bruker WM 250 spectrometer) usually in $CDCl_3$ with $(CH_3)_4Si$ as internal standard. Chemical shifts are given as δ units. Elemental analyses were performed by Mag. J. Theiner, microanalytical laboratory at the University of Vienna, Institute of Physical Chemistry.

General Procedure for the Synthesis of Amino Compounds 2–5. Synthesis of 5-[[(2,5-Dimethoxyphenyl)-amino]methyl]-2-hydroxybenzoic Acid Methyl Ester (6). 2,5-Dimethoxyaniline (200 mg, 1.3 mmol) and 5-formylsalicylic acid methyl ester (235 mg, 1.3 mmol) were dissolved in methanol (8 mL), and the mixture was heated to reflux overnight. Then sodium cyanoborohydride (245 mg, 3.9 mmol) was added in portions at room temperature to the stirred mixture. After stirring for an additional 4 h, the mixture was poured into 2 M aqueous pH 7 buffer solution and extracted with ethyl acetate. The extracts were combined, dried over magnesium sulfate, and concentrated in vacuo. The residue was twice chromatographed on silica gel (toluene/ethyl acetate = 5/1) to give pure **6** (213 mg, 51%) as a colorless oil: NMR δ 10.7 (s, 1H), 7.85 (d, J = 2.3 Hz, 1H), 7.48 (dd, J = 2.3 + 8.5 Hz, 1H), 6.96 (d, J = 8.5 Hz, 1H), 6.64–6.71 (m, 1H), 6.13–6.21 (m, 2H), 4.58 (br s, 1H), 4.24 (s, 2H), 3.94 (s, 3H), 3.81 (s, 3H), 3.72 (s, 3H). Anal. ($C_{17}H_{19}NO_5$) C, H, N.

5-[*N*-[(2,5-Dimethoxyphenyl)methyl]amino]-2-hydroxybenzoic acid (4): colorless crystals (from ethanol/water); mp 203 °C dec; NMR (CD_3OD/NaOD) δ 7.0 (d, J = 3 Hz, 1H), 6.71–6.94 (m, 4H), 6.57 (d, J = 8.3 Hz, 1H), 4.14 (s, 2H), 3.81 (s, 3H), 3.72 (s, 3H). Anal. ($C_{16}H_{17}NO_5$) C, H, N.

5-[*N*-[(2,5-Dihydroxyphenyl)methyl]amino]-2-hydroxybenzoic acid (2): yellowish crystals (from ethanol/water); mp 195–200 °C dec (lit.[10] mp ≥245 °C dec).

5-[*N*-[(2,5-Dihydroxyphenyl)methyl]amino]-2-hydroxybenzoic acid methyl ester (3): colorless crystals (from ethanol/water); mp 185 °C (lit.[10] mp 194–195 °C).

5-[*N*-[(2,5-Dimethoxyphenyl)methyl]amino]-2-hydroxybenzoic acid methyl ester (5): colorless crystals (from ethyl acetate/hexane); mp 88 °C (lit.[10] mp 79–80 °C).

Synthesis of 5-[(2,5-Dimethoxybenzyl)oxy]-2-hydroxybenzoic Acid Methyl Ester (8). Potassium carbonate (1.65 g, 12 mmol) was added to a solution of 2,5-dihydroxybenzoic acid methyl ester (2 g, 12 mmol) in dry acetone (50 mL). After addition of a solution of 2,5-dimethoxybenzyl bromide (2.73 g, 12 mmol) in dry acetone (20 mL), the mixture was heated to reflux for 30 h under vigorous stirring. Then the solvent was distilled off in vacuo and the residue partitioned between water and ethyl acetate. The organic layer was separated, dried over magnesium sulfate, and concentrated in vacuo. The crude product was purified by chromatography on silica gel (toluene/hexane = 10/1) followed by crystallization from ethanol yielding **8** (2.4 g, 64%) as colorless crystals: mp 110 °C; NMR δ 10.37 (s, 1H), 7.43 (d, J = 3 Hz, 1H), 7.16 (dd, J = 3 + 9 Hz, 1H), 7.05 (d, J = 2.5 Hz, 1H), 6.91 (d, J = 9 Hz, 1H), 6.78–6.86 (m, 2H), 5.04 (s, 2H), 3.95 (s, 3H), 3.83 (s, 3H), 3.77 (s, 3H); MS (EI) 318 (M^-), 287, 151, 136, 121. Anal. ($C_{17}H_{18}O_6$) C, H.

Synthesis of 5-[(2,5-Dimethoxyphenoxy)methyl]-2-hydroxybenzoic Acid Methyl Ester (9). A solution of 2,5-dimethoxyphenol[16] (300 mg, 1.94 mmol) in dry dimethylformamide (15 mL) was treated with sodium hydride (61 mg, 2.1 mmol, 80% in mineral oil) and stirred for 30 min at room temperature. Then a solution of 2-acetoxy-5-(bromomethyl)-benzoic acid methyl ester[17] (558 mg, 1.94 mmol) in dry dimethylformamide (8 mL) was added slowly, and the mixture was stirred for an additional 2 h. Aqueous workup and extraction with ethyl acetate afforded a mixture of the *O*-acetyl and a minor amount of the deacetylated product. Treatment of the crude product mixture with saturated aqueous sodium carbonate solution (2 mL) in methanol (20 mL) for 2 h at room temperature, followed by chromatographic purification (silica gel, toluene/ethyl acetate = 12/1), gave **9** as colorless crystals: mp 78–80 °C; NMR δ 10.77 (s, 1H), 7.92 (d, J = 2.3 Hz, 1H), 7.54 (dd, J = 2.3 + 8.5 Hz, 1H), 6.98 (d, J = 8.5 Hz, 1H), 6.8 (d, J = 8.8 Hz, 1H), 6.54 (d, J = 2.8 Hz, 1H), 6.42 (dd, J = 2.8 + 8.8 Hz, 1H), 5.0 (s, 2H), 3.93 (s, 3H), 3.82 (s, 3H), 3.72 (s, 3H). Anal. ($C_{17}H_{18}O_6$) C, H.

Synthesis of 5-[2-(2,5-Dimethoxyphenyl)ethyl]-2-hydroxybenzoic Acid Methyl Ester (13). (a) (*E*)- and (*Z*)-5-[2-(2,5-Dimethoxyphenyl)ethenyl]-2-hydroxybenzoic Acid Methyl Ester (10 and 11). At −30 °C, *n*-butyllithium (0.69 mL, 6.9 mmol, 10 M solution in hexane; Aldrich Chemical Co.) was added to a solution of diisopropylamine (0.69 g, 6.9 mmol) in dry tetrahydrofuran (20 mL). After stirring for an additional 30 min, the mixture was cooled to −50 °C, and (2,5-dimethoxybenzyl)triphenylphosphonium bromide (1.36 g, 2.75 mmol, prepared from 2,5-dimethoxybenzylbromide and triphenylphosphine in toluene) was added as a solid in three portions at this temperature. The orange suspension was stirred for 1 h at −50 °C, cooled to −70 °C, and treated with a solution of 5-formylsalicylic acid methyl ester (495 mg, 2.75

mmol) in dry tetrahydrofuran (10 mL). The mixture was stirred for 1 h at −70 °C and for 16 h after removal of the cooling bath and then poured into aqueous ammonium chloride solution. Extraction with ethyl acetate yielded a product mixture, which was chromatographed on silica gel (toluene/ethyl acetate = 4/1) to give 11 (198 mg, 23%) followed by its *E*-isomer **10** (376 mg, 43.5%). **11**: colorless crystals; mp 84–85 °C from ethanol; NMR δ 10.71 (s, 1H), 7.76 (d, J = 2 Hz, 1H), 7.35 (dd, J = 2 + 8.5 Hz, 1H), 6.72–6.86 (m, 4H), 6.62 (d, J = 12 Hz, 1H), 6.53 (d, J = 12 Hz, 1H), 3.9 (s, 3H), 3.78 (s, 3H), 3.57 (s, 3H). Anal. ($C_{18}H_{18}O_5$) C, H. **10**: colorless crystals; mp 85–87 °C from ethanol; NMR δ 10.78 (s, 1H), 7.97 (d, J = 2 Hz, 1H), 7.71 (dd, J = 2 + 8.5 Hz, 1H), 7.34 (d, J = 16 Hz, 1H), 7.13 (d, J = 3 Hz, 1H), 7.03 (d, J = 16 Hz, 1H), 7.0 (d, J = 8.5 Hz, 1H), 6.76–6.88 (m, 2H), 3.98 (s, 3H), 3.86 (s, 3H), 3.82 (s, 3H). Anal. ($C_{18}H_{18}O_5$) C, H.

(b) 5-[2-(2,5-Dimethoxyphenyl)ethyl]-2-hydroxybenzoic Acid Methyl Ester (13). Compound **10** or **11** or a mixture thereof (600 mg, 1.9 mmol) was dissolved in ethyl acetate (20 mL) and hydrogenated over palladium (40 mg, 10% on charcoal) for 16 h at atmospheric pressure. The mixture was filtered over Celite, and the filtrate was concentrated in vacuo. The residue was crystallized from ethanol to afford **13** (551 mg, 91%) as colorless crystals: mp 67 °C; NMR δ 10.6 (s, 1H), 7.67 (d, J = 2 Hz, 1H), 7.29 (dd, J = 2 + 8.5 Hz, 1H), 6.9 (d, J = 8.5 Hz, 1H), 6.66–6.83 (m, 3H), 3.95 (s, 3H), 3.78 (s, 3H), 3.74 (s, 3H), 2.73–2.9 (m, 4H), MS (EI) 316 (M^+), 285, 165, 151, 133, 121. Anal. ($C_{18}H_{20}O_5$) C, H.

General Procedure for the Synthesis of Salicylic Acid Analogs 7 and 12 by Ester Hydrolysis. 5-[(2,5-Dimethoxybenzyl)oxy]-2-hydroxybenzoic Acid (7). 5-[(2,5-Dimethoxybenzyl)oxy]-2-hydroxybenzoic acid methyl ester (120 mg, 0.38 mmol; **8**) was dissolved in methanol (10 mL) and treated with 1 N aqueous sodium hydroxide solution (1 mL, 1 mmol). The mixture was heated to reflux for 2 h and then concentrated in vacuo to remove the methanol. The residue was diluted with water and extracted with ethyl acetate to separate the starting material. The aqueous layer was acidified to pH 3 by treatment with 1 N aqueous hydrochloric acid and again extracted with ethyl acetate. The combined extracts were dried over magnesium sulfate and evaporated in vacuo to give **7** (92 mg, 80%) as colorless crystals: mp 128–131 °C; NMR (CD_3OD) δ 7.46 (d, J = 3.1 Hz, 1H), 7.19 (dd, J = 3.1 + 9 Hz, 1H), 7.03 (d, J = 2.9 Hz, 1H), 6.81–6.98 (m, 3H), 5.05 (s, 2H), 3.86 (s, 3H), 3.77 (s, 3H). Anal. ($C_{16}H_{16}O_6$) C, H.

5-[2-(2,5-Dimethoxyphenyl)ethyl]-2-hydroxybenzoic Acid (12). Starting with **13** (450 mg, 1.42 mmol), the same procedure followed by crystallization from toluene afforded **12** (335 mg, 78%) as colorless crystals: mp 138 °C; NMR δ 10.1 (br s), 7.74 (d, J = 2.3 Hz, 1H), 7.35 (dd, J = 2.3 + 8.5 Hz, 1H), 6.92 (d, J = 8.5 Hz, 1H), 6.79 (d, J = 8.6 Hz, 1H), 6.72 (dd, J = 3 + 8.6 Hz, 1H), 6.67 (d, J = 3 Hz, 1H), 3.78 (s, 3H), 3.75 (s, 3H), 2.77–2.94 (m, 4H). Anal. ($C_{17}H_{18}O_5$) C, H.

General Procedure for the Synthesis of Hydroquinone Compounds 14 and 15 by Ether Cleavage. 5-[2-(2,5-Dihydroxyphenyl)ethyl]-2-hydroxybenzoic Acid Methyl Ester (15). A solution of **13** (1.5 g, 4.74 mmol) in dry dichloromethane (25 mL) was cooled to −70 °C and treated slowly with a solution of boron tribromide (1.8 mL, 18.7 mmol) in dry dichloromethane (10 mL). The mixture was stirred for 30 min at −70 °C and then allowed to warm to room temperature. After stirring for 40 min at room temperature, the mixture was precooled to −20 °C and hydrolyzed with ice water. Extraction with ethyl acetate, drying over magnesium sulfate, and removal of the solvent gave a crude product, which was purified by filtration over silica gel (hexane/ethyl acetate = 1/1) to afford **15** (1.18 g, 86%) as colorless crystals: mp 163–164 °C; NMR (CD_3OD) δ 7.68 (d, J = 2.3 Hz, 1H), 7.34 (dd, J = 2.3 + 8.5 Hz, 1H), 6.89 (d, J = 8.5 Hz, 1H), 6.65 (dd, J = 1.4 + 7.5 Hz, 1H), 6.46–6.54 (m, 2H), 3.97 (s, 3H), 2.73–2.89 (m, 4H). Anal. ($C_{16}H_{16}O_5$) C, H.

5-[2-(2,5-Dihydroxyphenyl)ethyl]-2-hydroxybenzoic Acid (14). Starting with **12** (245 mg, 0.8 mmol), the same procedure afforded crude **14**, which was contaminated with residual boron complexes or boron oxides. The crude product was therefore dissolved in ethyl acetate and stirred vigorously with a 10% solution of glycerol in water for 30 min. The organic layer was separated, washed several times with water, dried over magnesium sulfate, and evaporated in vacuo to give pure **14** as orange crystals: mp 189–193 °C (lit.[9] mp 188–190 °C).

Biological Experiments. HaCaT Keratinocyte Proliferation Assay. HaCaT cells[18] were cultivated in DMEM (Gibco) containing 5% FCS as previously described.[19] For the proliferation assay, cells were detached by trypsinization, suspended in fresh medium, and seeded into 96-well microtiter plates at 4000 cells/0.2 mL/well. After 24 h, the medium was replaced with fresh medium containing graded concentrations of test compound. After 3–4 days of incubation, the extent of cellular proliferation was measured by a colorimetric assay using sulforhodamine B.[20] The results represent the average of at least two independent measurements.

EGF Receptor Tyrosine Kinase Assay. The assay mixture (30 μL total) contained 0.3 μM mouse EGF, solubilized EGF receptors from A431 human vulva carcinomas, 5 μM ATP, ca. 10^6 cpm [^{32}P]ATP, 12 mM $MgCl_2$, 2 mM $MnCl_2$, 0.1 mM sodium orthovanadate, 0.02 mM *p*-nitrophenyl phosphate, 0.3 mM tyrosyl peptide substrate (Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly), and graded concentrations of the test compounds dissolved at 100 mM in ethanol/Tween 80 (9/1) and appropriately diluted in assay buffer (0.1% Triton X-100, 10% glycerol, 150 mM NaCl, 50 mM Hepes, pH 7.6). The kinase was activated on ice by a 20-min preincubation with EGF prior to the addition of substrates to start the phosphorylation of the tyrosyl peptide (8 min). Following termination of the reaction by incubation with TCA (10%) and BSA (1%) for 30 min on ice, the protein was pelleted by centrifugation, and the phosphorylated peptide in the supernatant was adsorbed to P81 Whatman paper (2 × 2 cm). This paper was thoroughly washed three times in 75 mM phosphoric acid, containing 10 mM sodium pyrophosphate, followed by a final washing using acetone. Adsorbed radioactivity was quantified using a Packard Tri-Carb 2000CA analyzer.

Tumor Cell Proliferation. Tumor cell lines were grown at 37 °C under optimal medium and cell concentration conditions in the absence of antibiotics. At the time of exponential growth for tumor cell lines growing in suspension or at the time of 60–90% confluence for adherent cell lines, cells were harvested (adherent cells were trypsinized), suspended in fresh growth medium, and seeded into 96-well cell culture plates at concentrations ranging between 1000 and 5000 cells/well. Cells were grown at optimal initial concentration for 3–4 days in a final volume of 0.2 mL/well in the presence of graded concentrations of test compounds. Extent of cellular proliferation was measured by a colorimetric assay using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide[21] for cells growing in suspension or sulforhodamine B[20] for adherent cells.

References

(1) Parsons, J. T.; Parsons, S. J. Protein-tyrosine kinases, oncogenes, and cancer. *Important Adv. Oncol.* **1993**, 3–17.

(2) Nanney, L. B.; Stoscheck, C. M.; Magid, M.; King, L. E. Altered [^{125}I]epidermal growth factor binding and receptor distribution in psoriasis. *J. Invest. Dermatol.* **1986**, *86*, 260–265.

(3) Derynck, R.; Goeddel, D. V.; Ullrich, A.; Gutterman, J. U.; Williams, R. D.; Bringman, T. S.; Berger, W. H. Synthesis of messenger RNAs for transforming growth factors α and β and the epidermal growth factor receptor by human tumors. *Cancer Res.* **1987**, *47*, 707–712.

(4) Elder, J. T.; Fisher, G. J.; Lindquist, P. B.; Bennet, G. L.; Pittelkow, M. R.; Coffey, R. J.; Ellingsworth, L.; Derynck, R.; Voorhees, J. J. Overexpression of transforming growth factor α in psoriatic epidermis. *Science* **1989**, *243*, 811–814.

(5) Finzi, E.; Harkins, R.; Horn, T. TGF-α is widely expressed in differentiated as well as hyperproliferative skin epithelium. *J. Invest. Dermatol.* **1991**, *96*, 328–332.

(6) Onoda, T.; Iinuma, H.; Sasaki, Y.; Hamada, M.; Isshiki, K.; Naganawa, H.; Takeuchi, T.; Tatsuta, K.; Umezawa, K. Isolation of a novel tyrosine kinase inhibitor, lavendustin A, from Streptomyces griseolavendus. *J. Nat. Prod.* **1989**, *52*, 1252–1257.

(7) Onoda, T.; Isshiki, K.; Takeuchi, T.; Tatsuta, K.; Umezawa, K. Inhibition of tyrosine kinase and epidermal growth factor receptor internalization by lavendustin A methyl ester in cultured A431 cells. *Drugs Exp. Clin. Res.* **1990**, *16* (6), 249–253.

(8) Smyth, M. S.; Stefanova, I.; Horak, I. D.; Burke, T. R. J. Hydroxylated 2-(5'-salicyl)naphthalenes as protein-tyrosine kinase inhibitors. *J. Med. Chem.* **1993**, *36*, 3015–3020.
(9) Smyth, M. S.; Stefanova, I.; Hartmann, F.; Horak, I. D.; Osherov, N.; Levitzky, A.; Burke, T. R. J. Non-amine based analogues of lavendustin A as protein-tyrosine kinase inhibitors. *J. Med. Chem.* **1993**, *36*, 3010–3014.
(10) Chen, H.; Boiziau, J.; Parker, F.; Maroun, R.; Tocque, B.; Roques, B. P.; Garbay-Jaureguiberry, C. Synthesis and structure-activity studies of a series of [(hydroxybenzyl)amino]salicylates as inhibitors of EGF receptor-associated tyrosine kinase activity. *J. Med. Chem.* **1993**, *36*, 4094–4098.
(11) Chen, H.; Boiziau, J.; Parker, F.; Mailliet, P.; Commercon, A.; Tocque, B.; Le Pecq, J.-B.; Roques, B. P.; Garbay, C. Structure-activity relationships in a series of 5-[(2,5-dihydroxybenzyl)-amino]salicylate inhibitors of EGF-receptor associated tyrosine kinase: importance of additional hydrophobic aromatic interactions. *J. Med. Chem.* **1994**, *37*, 845–859.
(12) Winiski, A. P.; Meingassner, J. G. A novel antiproliferative agent derived from the tyrosine kinase inhibitor lavendustin A: mechanism of action. Poster presentation at the 1994 Annual Meeting of the Society for Investigative Dermatology, Baltimore, MD. *J. Invest. Dermatol.* **1994**, *102*, 565, Abstract 251.
(13) Nussbaumer, P.; Winiski, A. P.; Meingassner, J. G.; Cammisuli, S.; Weckbecker, G.; Stuetz, A. A novel antiproliferative agent derived from the tyrosine kinase inhibitor lavendustin A. Poster presentation at the 1994 Annual Meeting of the Society for Investigative Dermatology, Baltimore, MD. *J. Invest. Dermatol.* **1994**, *102*, 565, Abstract 252.
(14) Cammisuli, S.; Nussbaumer, P.; Winiski, A. P.; Stuetz, A.; Weckbecker, G. SDZ 281-977: a modified partial structure of lavendustin A that exerts potent and selective antiproliferative activities *in vitro* and *in vivo*. *Cancer Res.*, submitted for publication.
(15) Shulgin, A. T.; Gal, E. M. A new synthesis of 2,5-dihydroxyphenyl-DL-alanine adapted to isotopic scale. *J. Chem. Soc.* **1953**, 1316–1318.
(16) Syper, L. The Baeyer-Villiger oxidation of aromatic aldehydes and ketones with hydrogen peroxide catalyzed by selenium compounds. A convenient method for the preparation of phenols. *Synthesis* **1989**, 167–172.
(17) Regnier, G.; Canevari, R.; Le Douarec, J.-C. Acid-phenols. I. Synthesis of piperazine derivatives of salicylic acid. *Bull. Chem. Soc. Fr.* **1966**, *9*, 2821–2827.
(18) Boukamp, P.; Petrussevska, R. T.; Breitkreutz, D.; Hornung, J.; Markham, A.; Fusenig, N. E. Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. *J. Cell Biol.* **1988**, *106*, 761–771.
(19) Winiski, A. P.; Foster, C. A. ICAM-1 expression in a spontaneously transformed human keratinocyte cell line: characterization by a simple cell-ELISA assay. *J. Invest. Dermatol.* **1992**, *99*, 48–52.
(20) Skehan, P.; Storeng, R.; Scudiero, D.; Monks, A.; McMahon, J.; Vistica, D.; Warren, J. T.; Bokesch, H.; Kenney, S.; Boyd, M. R. New colorimetric cytotoxicity assay for anticancer-drug screening. *J. Natl. Cancer Inst.* **1990**, *82*, 1107–1112.
(21) Alley, M. C.; Scudiero, D. A.; Monks, A.; Hursey, M. L.; Czerwinski, M. J.; Fine, D. L.; Abbot, B. J.; Mayo, J. G.; Shoemaker, R. H.; Boyd, M. R. Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. *Cancer Res.* **1988**, *48*, 589–601.