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Structure–activity relationship studies on a novel class of antiproliferative agents derived from Lavendustin A. Part I: Ring A modifications

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ABSTRACT

The potent antiproliferative agent SDZ LAP 977, which has shown efficacy in a clinical proof of concept study in actinic keratosis patients, has been previously demonstrated to block the cell cycle in mitosis. In the present study, we further explored the mode of action: SDZ LAP 977 binds to the "colchicine binding site" on tubulin and, thus, inhibits tubulin polymerization in vitro. Moreover, we established structure–activity relationships for the effect of modifications in the 2,5-dimethoxyphenyl moiety ("ring A") of the molecule on in vitro antiproliferative activity.

1. Introduction

During a medicinal chemistry program on the natural product Lavendustin A, we discovered the novel antiproliferative agent 5-[2-(2,5-dimethoxyphenyl)ethyl]-2-hydroxybenzoic acid methyl ester (**1**, SDZ LAP 977; Fig. 1).[1] Compound **1** potently inhibits the proliferation of the human keratinocyte cell line HaCaT and several tumor cell lines in vitro; it was shown to be effective in vivo in a model of epidermal hyperplasia after topical application[2] and in selected tumor models after oral and intravenous administration[3]; furthermore, **1** showed efficacy in a clinical proof-of-concept study in actinic keratosis patients.[4] In contrast to Lavendustin A and the fully demethylated SDZ LAP 977 analog **2** (Fig. 1), which are both potent inhibitors of the epidermal growth factor receptor-associated tyrosine kinase,[1,5,6] compound **1** acts by blocking the cell cycle in mitosis.[1,3,7] Investigations on the antimitotic effect revealed that the block of the cell cycle in mitosis is due to interference of **1** with the microtubules of the mitotic spindle apparatus. New results on the mode of action are presented and discussed in this manuscript.

Next, we established detailed structure–activity relationships (SAR) based on the inhibitory potencies of the compounds on the proliferation of the human keratinocyte cell line HaCaT and spot-checked with selected compounds for the antimitotic mode of action. For easier interpretation of SAR results, the lead molecule **1** was dissected into three parts (Fig. 1): the two aromatic ring systems (rings A and B) and the spacer connecting them. In general, we found good correlation between series of analogs with the same modification in one part of the molecule, but differing at other sites of the molecule. The present manuscript describes the synthesis and the SAR of compounds with modifications in ring A.

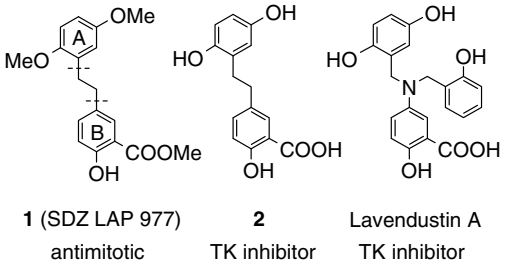

Figure 1. Structures and mode of action of Lavendustin A compared to derivatives **1** and **2**.

2. Chemistry

Several methods for the synthesis of **1** and analogs were developed (Scheme 1; for substitution pattern and compound number assignment of test compounds see also Table 1). The most generally applicable procedure was coupling of the two aromatic ring systems (rings A and B) by a Wittig reaction, followed by catalytic hydrogenation of the stilbene intermediates (Method A, Scheme 1). This route was compatible with various substituents, and, as starting materials, A- and B-ring derivatives could optionally be used either as phosphonium (**3**) or as benzaldehyde components (**4**).

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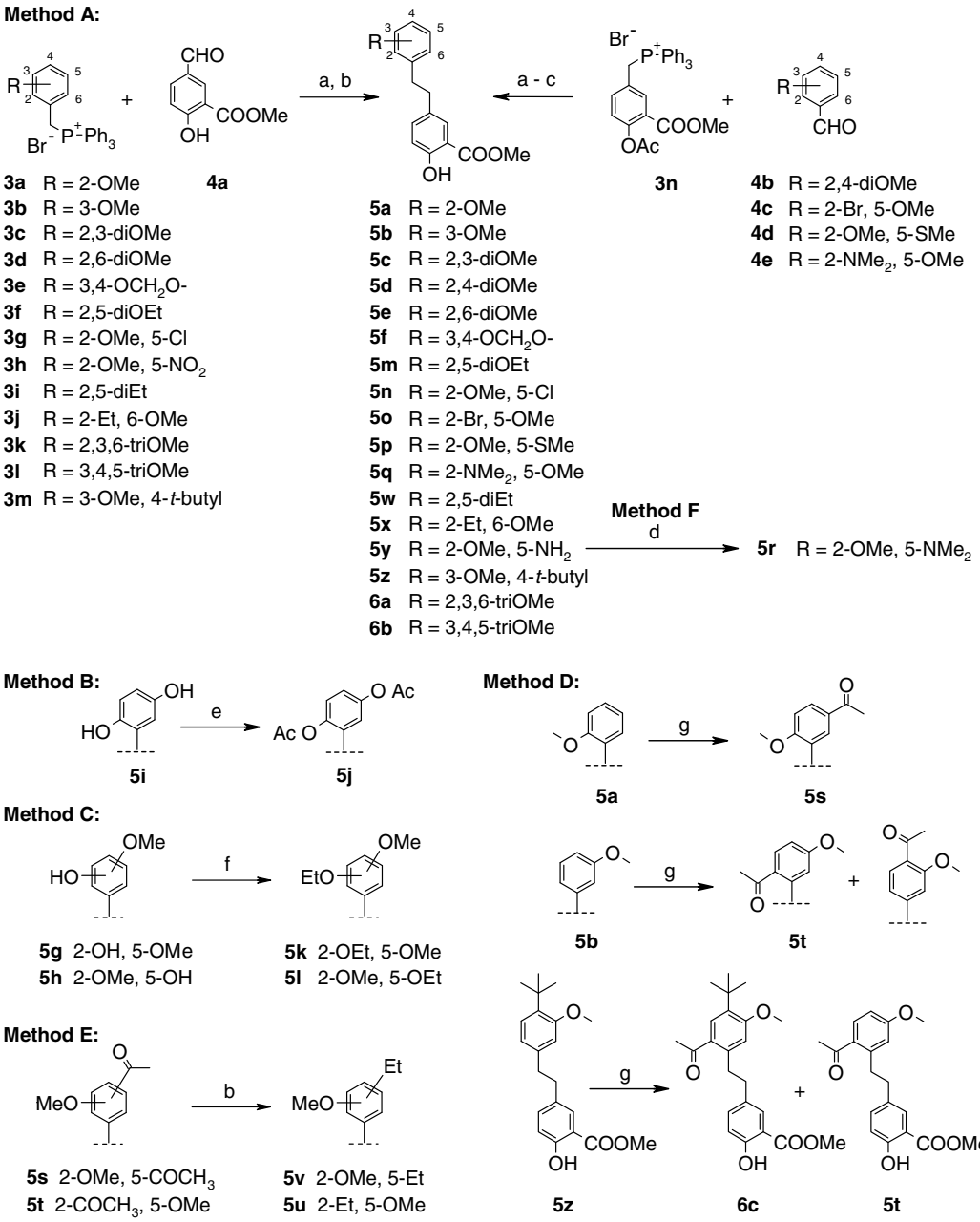
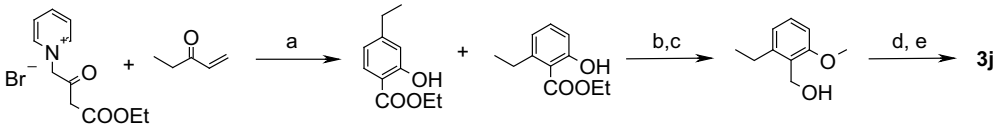


Scheme 1. Methods A–F used for the synthesis of test compounds **5** and **6**. Reagents: (a) LDA/THF; (b) H_2/Pd/C; (c) H^+ or OH^-; (d) CH_2O/NaH_2PO_3; (e) Ac_2O/Et_3N; (f) K_2CO_3/EtI; (g) AcCl/$AlCl_3$.

As reported for the synthesis of compound **1**,[1] unprotected 5-formylsalicylic acid methylester (**4a**) could be used successfully in the Wittig reaction, when lithium diisopropylamide (LDA) in tetrahydrofuran prepared from a highly concentrated solution of *n*-butyllithium (10 M solution in hexane, Sigma–Aldrich) was employed as base to deprotonate the phosphonium salts **3a–m**. The use of other bases or a higher percentage of hexane in the solvent mixture (e.g., resulting 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. Alternatively, a protection/deprotection protocol for the salicylic hydroxy group in the starting material could be applied, as it was exercised when the phosphonium component **3n** was the starting material in the Wittig reaction (Scheme 1). In these cases only a slight excess of LDA (1.2 equiv) was used, in contrast to the reactions starting from **4a** (3 equiv of LDA). The *O*-acetyl protecting group resulting from **3n** was either removed by acidic (2 N HCl) or mild basic hydrolysis (in some experiments the *O*-acetyl group was already partially cleaved during the Wittig reaction and work-up). Different *E*/*Z*-ratios as well as varying yields of stilbenes were obtained from the Wittig reaction, depending on the substitution pattern. For instance, the *E*-isomer was almost exclusively formed in 2,6-disubstituted derivatives. In some cases, the pure, single *E*- and *Z*-isomers could be isolated by chromatography on silica gel, but usually they were only separated from the triphenylphosphine oxide and other reaction by-products and used as mixture in the subsequent hydrogenation step. *n*-Butylesters of the *E*/*Z*-stilbenes were identified as by-products in several experiments, in particular, when LDA was prepared from 10 M of *n*-butyllithium. The formation of the *n*-butylesters was probably due to transesterification of the corresponding methylesters by lithium butoxide, an impurity in the butyllithium



Scheme 2. Synthetic route toward **3j**, intermediate in the synthesis of **5x**. Reagents: (a) Et_3N; (b) NaH/MeI; (c) DiBAH/toluene; (d) DiPhos/Br_2; (e) Ph_3P/toluene.

reagent generated by air oxidation. Novartis Process Research and Development developed a reproducible plant process for the production of **1** using a Horner–Wadsworth–Emmons-type olefination as the key step and without the need for a column chromatography.[8] When the stilbene product resulting from the Wittig reaction of 3 h and **4a** was hydrogenated, the double bond and the nitro group were reduced concomitantly to give **5y** which was not tested but further converted to **5r**.

Several derivatives were prepared from other test compounds by straightforward functional group transformations. This includes O-acetylation of **5i** to produce compound **5j** (Method B, Scheme 1), selective O-alkylation in **5g,h** to give the ethoxy analogs **5k,l** (Method C, Scheme 1), catalytic hydrogenation of acetophenones **5s,t** over palladium on charcoal in the preparation of analogs **5u,v** (Method E, Scheme 1), and N,N-dimethylation of the amino function in **5y** to yield substance **5r** (Method F, Scheme 1).

Methoxyacetophenones **5s,t** were generally synthesized by Friedel–Crafts acylation (Method D, Scheme 1). Whereas the reaction of **5a** with acetyl chloride/aluminum chloride afforded the respective acetylated analogs **5s** in good yield, the same procedure gave a mixture of two isomeric products (2-acetyl-5-methoxy and 4-acetyl-3-methoxy) when starting from **5b**. The isomers could not be easily separated, and the desired *para*-substituted product **5t** could only be isolated in small amount after selective Lewis acid-mediated transformation of the *o*-methoxyacetophenone into the corresponding *o*-hydroxyacetophenone by chromatographic separation from this new product. For the synthesis of a larger sample of this biologically interesting substance, a new method was developed using the *t*-butyl group as protecting group (Scheme 1). The *t*-butyl group was chosen to block the *o*-position against acetylation, thus, avoiding the formation of the unwanted regioisomer. Furthermore, we anticipated it to be cleaved afterwards by a retro-Friedel–Crafts alkylation reaction. Thus, compound **5z**, prepared via Method A, was treated with 3 equiv of acetyl chloride and aluminum chloride under usual Friedel–Crafts acylation conditions. Two products were isolated: compound **6c** from the acetylation reaction and the desired analog **5t** formed by subsequent dealkylation of **6c**. The formation of **5t** under the reaction conditions confirmed the envisaged strategy and indicated the feasibility of a one-pot procedure for its synthesis. Several additional experiments were run at various reaction temperatures and with different molar equivalents of aluminum chloride. The optimum reaction conditions for the one-pot conversion of **5z** into **5t** were found to be rapid addition of 4 equiv of aluminum chloride to a solution of 1 equiv of **5z** and 2.2 equiv of acetyl chloride in dichloroethane without cooling, followed by maintaining the reaction temperature at about 35 °C for 1 h by external heating. Thus, **5t** was obtained directly from **5z** in 61% isolated yield, which was even superior to the yield obtained for the corresponding two-step procedure, that is, in synthesis of **6c** under milder conditions (85% yield) and subsequent transformation into **5t** by treatment with aluminum chloride (68% yield).

The key starting materials for all test compounds were either commercially available or synthesized in analogy to standard procedures (see Section 4). For the preparation of a precursor of phosphonium salt **3j**, a procedure reported for the synthesis of alkylaryl disubstituted ethyl salicylates[9] was adapted. Thus, 1-(3-ethoxycarbonyl-2-oxopropyl)-pyridinium bromide was reacted with ethyl vinyl ketone in the presence of triethylamine to give a mixture of the regioisomeric 4-ethyl- and 6-ethylsalicylates (Scheme 2), which were separated by chromatography on silica gel. The 6-ethyl product was then transformed into **3j** by four conventional steps.

Some characteristic physicochemical properties of the new Lavendustin derivatives **5** and **6** are listed in Table 2.

3. Results and discussion of biological activities

A previous study focusing on the structural requirements for the switch in mode of action from the tyrosine kinase inhibitor Lavendustin A to **1** revealed that the presence of one methoxy group in the 2,5-disubstituted ring A is sufficient for inducing mitotic arrest of keratinocytes.[7] Already from this limited study, a trend for the essential elements needed for potent antiproliferative activity of **1**-related analogs was perceivable. For deeper insight and the establishment of detailed SAR, series of additional analogs were synthesized and tested for antiproliferative activity in the keratinocyte cell line HaCaT. In addition, their antimitotic potential was determined to confirm the antimitotic mode of action. The present manuscript deals with the SAR obtained by modifications in ring A.

The first series of derivatives (**5a–f**) was designed in order to probe the importance of the position of the methoxy substituents in **1** for antiproliferative activity. Both analogs with a single methoxy group in positions 2 and 3 (numbering with respect to the position of the spacer), respectively, show drastically reduced antiproliferative activity (**5a** and **5b**, Table 1). Despite their low antiproliferative activity, both monomethoxy compounds are also antimitotic. The results obtained for the 2,3-dimethoxy (**5c**), the 2,4-dimethoxy (**5d**), the 2,6-dimethoxy (**5e**), and the 3,4-methylenedioxy analog (**5f**)—all possible variations of a dialkoxy substitution pattern of **1**—demonstrate that, in addition to the 2,5-, only the 2,6-disubstitution pattern is tolerated for high activity. With an IC_{50} value of 110 nM, the 2,6-dimethoxy analog **5e** is about 2-fold less active than **1**. Interestingly, those derivatives, which have the two (alk)oxy substituents in a neighboring position, that is, **5c** and **5f**, do not exert an antimitotic activity up to 100 μM. This finding is probably not a result of their poor antiproliferative potency or a matter of the detection limit of the assay, since with other derivatives induction of mitotic arrest is usually observed at concentrations near their antiproliferative IC_{50} values. For example, compound **5b** shows a similarly low antiproliferative activity as **5c** and **5f**, but, in contrast, a clear antimitotic activity below 100 μM. Obviously, compounds **5c** and **5f** exert their antiproliferative effects via another mechanism of action.

Analogs **5g–m** represent a subseries of compounds in which the 2,5- and 2,6-dioxy substitution pattern, respectively, is kept constant. As previously reported,[7] the monodemethyl derivatives of **1** (i.e., substances **5g,h**) are substantially less active than the parent compound but still antimitotic. When both methoxy groups are demethylated (**5i**), the antiproliferative potency is further reduced and the ability to block HaCaT cells in mitosis is lost. Thus,

Table 1
In vitro antiproliferative (IC_{50}) and antimitotic activity of Lavendustin derivatives **1**, **5**, and **6**

Compound	Substitution pattern					HaCaT cells		
	Pos. 2	Pos. 3	Pos. 4	Pos. 5	Pos. 6	Antiproliferative IC_{50} (nM)	Antimitotic activity	
							Qualitative[a]	Quantitative (μM)[b]
1	OMe	H	H	OMe	H	47	+	36% @ 0.2
5a	OMe	H	H	H	H	20,000	+	16% @ 30
5b	H	H	H	OMe	H	37,000	+	36% @ 100
5c	OMe	OMe	H	H	H	33,000	-	3.7% @ 100
5d	OMe	H	OMe	H	H	19,000	+	7.2% @ 100
5e	OMe	H	H	H	OMe	110	+	24% @ 0.4
5f	H	OCH_2O		H	H	36,000	–	2.9% @ 100
5g	OH	H	H	OMe	H	1100	+	27% @ 2
5h	OMe	H	H	OH	H	8700	+	57% @ 20
5i	OH	H	H	OH	H	21,000	–	2.2% @ 50
5j	OAc	H	H	OAc	H	24,000	n.d.	n.d.
5k	OEt	H	H	OMe	H	120	+	38% @ 0.2
5l	OMe	H	H	OEt	H	540	+	31% @ 0.5
5m	OEt	H	H	OEt	H	380	+	20% @ 3
5n	OMe	H	H	Cl	H	6900	+	27% @ 30
5o	Br	H	H	OMe	H	210	+	20% @ 1
5p	OMe	H	H	SMe	H	860	+	37% @ 2.5
5q	NMe_2	H	H	OMe	H	650	+	42% @ 3
5r	OMe	H	H	NMe_2	H	920	+	28% @ 2.5
5s	OMe	H	H	$COCH_3$	H	660	+	26% @ 2
5t	$COCH_3$	H	H	OMe	H	41	+	46% @ 1.5
5u	Et	H	H	OMe	H	130	+	47% @ 0.4
5v	OMe	H	H	Et	H	430	+	44% @ 1
5w	Et	H	H	Et	H	650	+	30% @ 2
5x	OMe	H	H	H	Et	200	+	50% @ 1
6a	OMe	OMe	H		OMe	3500	+	25% @ 10
6b	H	OMe	OMe	OMe	H	3500	+	18% @ 10
6c	$COCH_3$	H	*t*-Bu	OMe	H	8200	–	4.4% @ 100

[a] Qualitative evaluation: +, antimitotic; –, not antimitotic up to 100 μM; n.d., not determined.
[b] Quantitative evaluation: % mitotic cells after 20 h incubation with indicated test concentration; controls of untreated cells were in the range of 1.4–4.7%.

compound **5i** does not share the same mode of action with **1** and its potent antiproliferative analogs. In contrast to dimethylation, diacetylation of the hydroquinone system of **5i** generating derivative **5j** does not lead to an increase in antiproliferative activity, and **5j** was found not to be antimitotic. Considering that compounds **5g,h,i** are potential metabolites of **1**, the in vitro results suggest that they do not contribute to the observed in vivo efficacy of SDZ LAP 977 and that substantial deactivation of **1** can be expected by such a metabolic degradation pathway.

Compounds **5k–m** with one or two of the methoxy groups in **1** replaced by the ethoxy substituent retain high antiproliferative and clear antimitotic activity. Nevertheless, with IC_{50} values ranging from 120 nM to 540 nM, these analogs are less potent than **1**. Consequently, sterically more demanding alkoxy substituents were not investigated. Within the subset of 2,5-di(alk)oxy analogs, antiproliferative activity seems to be more pronouncedly affected by changes at the 5-O versus the 2-O substituent, as can be seen by comparing the IC_{50} values of derivatives **5k** (2-OEt, 5-OMe; 120 nM) and **5g** (2-OH, 5-OMe; 1100 nM) with those of analogs **5l** (2-OMe, 5-OEt; 540 nM) and 5 h (2-OMe, 5-OH; 8700 nM). Additional pairs of analogs confirm this trend as a general feature of the SAR (vide infra).

Next, the influence of various other substituents—including halogens, methylthio, dimethylamino, acetyl, and alkyl residues—was thoroughly investigated by synthesizing and testing compounds **5n–5x**. The 2-bromo derivative **5o** shows high potency with an IC_{50} value of 210 nM, whereas the 5-chloro compound **5n** is only weakly active. Replacement of the 5-methoxy group in **1** by the methylthio (**5p**) or the dimethylamino substituent (**5r**), as well as the analogous modification at position 2 (**5q**), results in decreased antiproliferative potency by a similar factor of about 14–20. Interestingly, highest activity in the whole series is achieved by introduction of the acetyl substituent in position 2 (compound **5t**, IC_{50} value = 41 nM). Despite the obviously different electronic effects of the acetyl relative to the original methoxy group, compounds **5t** and **1** have very similar activity and the same mode of action. Analogous substitution by acetyl in position 5 of ring A yielding analog **5s**, however, caused a 15-fold reduction in potency. Derivatives **5u–x**, where ethyl groups replace one or two of the methoxy groups in **1** and its 2,6-isomer **5e,** exhibit again altered electronic properties and show high to moderate activity with IC_{50} values between 130 and 650 nM. The 5-ethyl compound **5v** is about 3-fold less potent than the 2-ethyl analog **5u**, and replacement of both methoxy groups in **1** by ethyl results in an additive loss of activity (**5w**). The 2,6-disubstituted compound **5x** exhibits strong antiproliferative activity, but is slightly less effective than the 2,5-disubstituted analog **5u**. Although compounds **5n–x** cover a broad range of antiproliferative activity (IC_{50} values ranging from 41 nM to 6.9 μM), they all exert the antimitotic mode of action as observed for **1**. The results obtained with the described disubstituted compounds suggest that the antiproliferative activity of Lavendustin derivatives is generally more sensitive to steric rather than electronic effects of the substituents in ring A.

Finally, a series of derivatives with three A-ring substituents were investigated (**6a–c**), in particular, to compare our compound class with another class of antimitotic agents, the combretastatins. The natural product combretastatin A4,[10] one of the most potent representatives, features a 3,4,5-trimethoxyphenyl and a 3-hydroxy-4-methoxyphenyl residue connected via a (*Z*)-ethenylene linker. Although this compound has some structural similarity to **1** and its derivatives, SAR differs substantially, as demonstrated, for example, by the weak activity (IC_{50} = 3.5 μM) of our 3,4,5-trimethoxy-substituted analog **6b**. As discussed above, both the 2,5-dimethoxy (**1**) and the 2,6-dimethoxy (**5e**) substitution patterns provide high antiproliferative potency. However, the 2,3,6-trimethoxy

Table 2
Physicochemical properties of new Lavendustin derivatives **5** and **6**

Compound	Morph.,[a] mp (°C)	NMR ($CDCl_3$) δ	Method[b]/% yield[c]
5a	Oil	10.59 (s, 1H), 7.67 (d, *J* = 2.3 Hz, 1H), 7.29 (dd, *J* = 2.3 + 8.5 Hz, 1H), 7.20 (dt, *J* = 1.8 + 7.8 Hz, 1H), 7.08 (dd, *J* = 1.8 + 7.8 Hz, 1H), 6.84-6.93 (m, 3H), 3.96 (s, 3H), 3.84 (s, 3H), 2.75-2.94 (m, 4H)	A/68
5b	Oil	10.60 (s, 1H), 7.66 (d, *J* = 2.3 Hz, 1H), 7.26 (dd, *J* = 2.3 + 8.5 Hz, 1H), 7.21 (t, *J* = 7.9 Hz, 1H), 6.91 (d, *J* = 8.5 Hz, 1H), 6.7–6.8 (m, 3H), 3.94 (s, 3H), 3.78 (s, 3H), 2.85 (br s, 4H)	A/56
5c	75–77	10.61 (s, 1H), 7.69 (d, *J* = 2.2 Hz, 1H), 7.30 (dd, *J* = 2.2 + 8.5 Hz, 1H), 6.95 (t, *J* = 8 Hz, 1H), 6.91 (d, *J* = 8.5 Hz, 1H), 6.80 (dd, *J* = 1 + 8 Hz, 1H), 6.74 (dd, *J* = 1 + 8 Hz, 1H), 3.95 (s, 3H), 3.88 (s, 3H), 3.84 (s, 3H), 2.78–2.97 (m, 4H)	A/78
5d	64–66	10.59 (s, 1H), 7.64 (d, *J* = 2.2 Hz, 1H), 7.26 (dd, *J* = 2.2 + 8.5 Hz, 1H), 6.96 (d, *J* = 8.2 Hz, 1H), 6.90 (d, *J* = 8.5 Hz, 1H), 6.46 (d, *J* = 2.3 Hz, 1H), 6.40 (dd, *J* = 2.3 + 8.2 Hz, 1H), 3.96 (s, 3H), 3.81 (s, 6H), 2.80 (br s, 4H)	A/76
5e	95–97	10.59 (s, 1H), 7.69 (d, *J* = 2.3 Hz, 1H), 7.32 (dd, *J* = 2.3 + 8.5 Hz, 1H), 7.15 (t, *J* = 8.3 Hz, 1H), 6.90 (d, *J* = 8.5 Hz, 1H), 6.54 (d, *J* = 8.3 Hz, 2H), 3.96 (s, 3H), 3.79 (s, 6H), 2.87–2.96 (m, 2H), 2.65–2.76 (m, 2H)	A/79
5f	89	10.60 (s, 1H), 7.63 (d, *J* = 2.3 Hz, 1H), 7.24 (dd, *J* = 2.3 + 8.5 Hz, 1H), 6.90 (d, *J* = 8.5 Hz, 1H), 6.72 (d, *J* = 7.8 Hz, 1H), 6.66 (d, *J* = 1.6 Hz, 1H), 6.59 (dd, *J* = 1.6 + 7.8 Hz, 1H), 5.93 (s, 2H), 3.95 (s, 3H), 2.80 (br s, 4H)	A/83
5j	Oil	10.61 (s, 1H), 7.62 (d, *J* = 2.3 Hz, 1H), 7.22 (dd, *J* = 2.3 + 8.5 Hz, 1H), 7.06 (d, *J* = 8.5 Hz, 1H), 6.97 (dd, *J* = 2.5 + 8.5 Hz, 1H), 6.94 (d, *J* = 2.5 Hz, 1H), 6.91 (d, *J* = 8.5 Hz, 1H), 3.94 (s, 3H), 2.78 (br.s, 4H), 2.31 (s, 3H), 2.28 (s, 3H)	B/39
5k	Oil	10.59 (s, 1H), 7.67 (d, *J* = 2.3 Hz, 1H), 7.28 (dd, *J* = 2.3 + 8.5 Hz, 1H), 6.90 (d, *J* = 8.5 Hz, 1H), 6.66–6.81 (m, 3H), 3.98 (qua, *J* = 7 Hz, 2H), 3.94 (s, 3H), 3.74 (s, 3H), 2.84 (br s, 4H), 1.38 (tr, *J* = 7 Hz, 3H)	C/36
5l	Oil	10.60 (s, 1H), 7.67 (d, *J* = 2.3 Hz, 1H), 7.29 (dd, *J* = 2.3 + 8.5 Hz, 1H), 6.91 (d, *J* = 8.5 Hz, 1H), 6.68–6.82 (m, 3H), 3.96 (qua, *J* = 7 Hz, 2H), 3.95 (s, 3H), 3.78 (s, 3H), 2.82 (br s, 4H), 1.41 (tr, *J* = 7 Hz, 3H)	C/49
5m	Oil	10.59 (s, 1H), 7.68 (d, *J* = 2.3 Hz, 1H), 7.29 (dd, *J* = 2.3 + 8.5 Hz, 1H), 6.92 (d, *J* = 8.5 Hz, 1H), 6.65-6.81 (m, 3H), 3.99 (qua, *J* = 7 Hz, 2H), 3.96 (s, 3H), 3.94 (qua, *J* = 7 Hz, 2H), 2.76–2.93 (m, 4H), 1.42 (tr, *J* = 7 Hz, 3H), 1.38 (tr, *J* = 7 Hz, 3H)	A/78
5n	59–62	10.60 (s, 1H), 7.65 (d, *J* = 2.3 Hz, 1H), 7.28 (dd, *J* = 2.3 + 8.5 Hz, 1H), 7.15 (dd, *J* = 2.6 + 8.6 Hz, 1H), 7.07 (d, *J* = 2.6 Hz, 1H), 6.91 (d, *J* = 8.5 Hz, 1H), 6.77 (d, *J* = 8.6 Hz, 1H), 3.95 (s, 3H), 3.80 (s, 3H), 2.74–2.90 (m, 4H)	A/66
5o	67–68	10.63 (s, 1H), 7.67 (d, *J* = 2.3 Hz, 1H), 7.43 (d, *J* = 8.5 Hz, 1H), 7.30 (dd, *J* = 2.3 + 8.5 Hz, 1H), 6.92 (d, *J* = 8.5 Hz, 1H), 6.69 (d, *J* = 3 Hz, 1H), 6.65 (dd, *J* = 3 + 8.5 Hz, 1H), 3.95 (s, 3H), 3.74 (s, 3H), 2.78–2.99 (m, 4H)	A[d]/58
5p	Oil	10.59 (s, 1H), 7.65 (d, *J* = 2.3 Hz, 1H), 7.27 (dd, *J* = 2.3 + 8.5 Hz, 1H), 7.18 (dd, *J* = 2.4 + 8.5 Hz, 1H), 7.07 (d, *J* = 2.3 Hz, 1H), 6.9 (d, *J* = 8.5 Hz, 1H), 6.81 (d, *J* = 8.5 Hz, 1H), 3.96 (s, 3H), 3.81 (s, 3H), 2.74–2.94 (m, 4H), 2.41 (s, 3H)	A/48
5q	Oil	10.60 (s, 1H), 7.69 (d, *J* = 2.3 Hz, 1H), 7.33 (dd, *J* = 2.3 + 8.5 Hz, 1H), 7.07–7.12 (m, 1H), 6.91 (d, *J* = 8.5 Hz, 1H), 6.72–6.76 (m, 2H), 3.95 (s, 3H), 3.76 (s, 3H), 2.82–3.02 (m, 4H), 2.62 (s, 6H)	A/59
5r	Oil	10.59 (s, 1H), 7.69 (d, *J* = 2.3 Hz, 1H), 7.32 (dd, *J* = 2.3 + 8.5 Hz, 1H), 6.92 (d, *J* = 8.5 Hz, 1H), 6.81 (d, *J* = 8.5 Hz, 1H), 6.63 (dd, *J* = 3 + 8.5 Hz, 1H), 6.60 (d, *J* = 3 Hz, 1H), 3.96 (s, 3H), 3.79 (s, 3H), 2.79–2.91 (m, 10H)	F/53
5s	107–108	10.60 (s, 1H), 7.86 (dd, *J* = 2.3 + 8.6 Hz, 1H), 7.74 (d, *J* = 2.3 Hz, 1H), 7.66 (d, *J* = 2.3 Hz, 1H), 7.28 (dd, *J* = 2.3 + 8.5 Hz, 1H), 6.91 (d, *J* = 8.5 Hz, 1H), 6.89 (d, *J* = 8.6 Hz, 1H), 3.96 (s, 3H), 3.90 (s, 3H), 2.77–2.95 (m, 4H), 2.54 (s, 3H)	D/64
5t	74–75	10.62 (s, 1H), 7.79 (d, *J* = 8.7 Hz, 1H), 7.71 (d, *J* = 2.3 Hz, 1H), 7.38 (dd, *J* = 2.3 + 8.5 Hz, 1H), 6.91 (d, *J* = 8.5 Hz, 1H), 6.79 (dd, *J* = 2.7 + 8.7 Hz, 1H), 6.69 (d, *J* = 2.7 Hz, 1H), 3.94 (s, 3H), 3.82 (s, 3H), 3.11–3.19 (m, 2H), 2.76–2.84 (m, 2H), 2.56 (s, 3H)	D/61[e]
5u	Oil	10.65 (s, 1H), 7.69 (d, *J* = 2.3 Hz, 1H), 7.30 (dd, *J* = 2.3 + 8.5 Hz, 1H), 7.13 (d, *J* = 8.3 Hz, 1H), 6.95 (d, *J* = 8.5 Hz, 1H), 6.75 (dd, *J* = 2.8 + 8.3 Hz, 1H), 6.73 (d, *J* = 2.8 Hz, 1H), 3.97 (s, 3H), 3.79 (s, 3H), 2.82–2.91 (m, 4H), 2.62 (qua, *J* = 7.5 Hz, 2H), 1.22 (t, *J* = 7.5 Hz, 3H)	E/85
5v	83–86	10.58 (s, 1H), 7.67 (d, *J* = 2.3 Hz, 1H), 7.29 (dd, *J* = 2.3 + 8.5 Hz, 1H), 7.02 (dd, *J* = 2.3 + 8.2 Hz, 1H), 6.91 (d, *J* = 2.3 Hz, 1H), 6.90 (d, *J* = 8.5 Hz, 1H), 6.79 (d, *J* = 8.2 Hz, 1H), 3.96 (s, 3H), 3.82 (s, 3H), 2.76–2.91 (m, 4H), 2.56 (qua, *J* = 7.6 Hz, 2H), 1.19 (t, *J* = 7.6 Hz, 3H)	E/93
5w	47	10.61 (s, 1H), 7.67 (d, *J* = 2.2 Hz, 1H), 7.29 (dd, *J* = 2.2 + 8.5 Hz, 1H), 7.13 (d, *J* = 7.8 Hz, 1H), 7.02 (dd, *J* = 1.8 + 7.8 Hz, 1H), 6.97 (d, *J* = 1.8 Hz, 1H), 6.93 (d, *J* = 8.5 Hz, 1H), 3.97 (s, 3H), 2.76–2.93 (m, 4H), 2.65 (qua, *J* = 7.5 Hz, 2H), 2.60 (qua, *J* = 7.6 Hz, 2H), 1.25 (t, *J* = 7.5 Hz, 3H), 1.23 (t, *J* = 7.6 Hz, 3H)	A/77
5x	Oil	10.62 (s, 1H), 7.7 (d, *J* = 2.3 Hz, 1H), 7.34 (dd, *J* = 2.3 + 8.5 Hz, 1H), 7.17 (t, *J* = 7.9 Hz, 1H), 6.94 (d, *J* = 8.5 Hz, 1H), 6.84 (d, *J* = 7.9 Hz, 1H), 6.76 (d, *J* = 7.9 Hz, 1H), 3.98 (s, 3H), 3.84 (s, 3H), 2.88–2.96 (m, 2H), 2.69–2.78 (m, 2H), 2.63 (qua, *J* = 7.5 Hz, 2H), 1.22 (t, *J* = 7.5 Hz, 3H)	A/63
5y	Oil, HCl: 172–175	10.59 (s, 1H), 7.67 (d, *J* = 2.3 Hz, 1H), 7.28 (dd, *J* = 2.3 + 8.5 Hz, 1H), 6.89 (d, *J* = 8.5 Hz, 1H), 6.70 (d, *J* = 8.3 Hz, 1H), 6.53 (dd, *J* = 2.8 + 8.3 Hz, 1H), 6.49 (d, *J* = 2.8 Hz, 1H), 3.95 (s, 3H), 3.75 (s, 3H), 2.79 (s, 4H)	A/55
5z	Oil	10.61 (s, 1H), 7.65 (d, *J* = 2.3 Hz, 1H), 7.30 (dd, *J* = 2.3 + 8.5 Hz, 1H), 7.19 (d, *J* = 7.8 Hz, 1H), 6.92 (d, *J* = 8.5 Hz, 1H), 6.73 (dd, *J* = 1.8 + 7.8 Hz, 1H), 6.66 (d, *J* = 1.8 Hz, 1H), 3.95 (s, 3H), 3.81 (s, 3H), 2.86 (br.s, 4H), 1.36 (s, 9H)	A/61
6a	87–90	10.60 (s, 1H), 7.70 (d, *J* = 2.3 Hz, 1H), 7.34 (dd, *J* = 2.3 + 8.5 Hz, 1H), 6.91 (d, *J* = 8.5 Hz, 1H), 6.74 (d, *J* = 8.9 Hz, 1H), 6.56 (d, *J* = 8.9 Hz, 1H), 3.96 (s, 3H), 3.83 (s, 3H), 3.80 (s, 3H), 3.76 (s, 3H), 2.70–2.95 (m, 4H)	A/73
6b	90	10.61 (s, 1H), 7.64 (d, *J* = 2.3 Hz, 1H), 7.27 (dd, *J* = 2.3 + 8.5 Hz, 1H), 6.92 (d, *J* = 8.5 Hz, 1H), 6.36 (s, 2H), 3.95 (s, 3H), 3.83 (s, 6H), 2.78–2.89 (m, 4H)	A/82
6c	114–117	10.62 (s, 1H), 7.74 (s, 1H), 7.69 (d, *J* = 2.3 Hz, 1H), 7.41 (dd, *J* = 2.3 + 8.5 Hz, 1H), 6.92 (d, *J* = 8.5 Hz, 1H), 6.59 (s, 1H), 3.94 (s, 3H), 3.83 (s, 3H), 3.09–3.17 (m, 2H), 2.78–2.86 (m, 2H), 2.58 (s, 3H), 1.39 (s, 9H)	D/85

[a] Morph., morphology.
[b] Method A consists of 2 steps (Wittig reaction and subsequent hydrogenation) and overall yields are given.
[c] Yields (not optimized) of isolated, analytically pure products.
[d] Rhodium (10% on charcoal) instead of Pd was used in the hydrogenation step for the synthesis of **5o**.
[e] Yield for two steps starting from **5z**.

analog **6a**, where both substitution patterns are merged into one structure, shows strongly reduced activity (IC_{50} = 3.5 μM). Addition of the bulky *t*-butyl residue to **5t** to yield **6c**, the most potent compound of the whole study, results in loss of the antimitotic mode of action. Due to the consistently weak activity of compounds **6a–c**, no further trisubstituted analogs were investigated.

Searching for the molecular basis of the antimitotic activity exhibited by this compound class, we initiated detailed investigations with **1**. In initial studies with HaCaT keratinocytes, we observed that mitotic arrest of cells occurred within a few hours resulting in large numbers of cells containing micronuclei after 1 day. The microtubules of the spindle apparatus were perturbed at the low, antiproliferative concentrations as visualized with indirect immunofluorescence using antibodies against α- and β-tubulin,[3] strongly suggesting either tubulin or microtubule-associated proteins as the molecular target. Dose-response curves revealed an excellent correlation between the antiproliferative and antimitotic effects. Effects on interphase microtubules were seen at much higher concentrations, that is, in the μM range.[11] Compound **1** was then tested in a tubulin polymerization assay in vitro and exhibited an IC_{50} of 1.0 μM (Fig. 2; podophyllotoxin was used as reference compound and gave an IC_{50} of 0.56 μM). Thus, with this activity, **1** is among the most potent known tubulin polymerization inhibitors. There are several defined binding sites on tubulin for antimitotic agents. Based on some structural similarity to known binders, we investigated whether **1** binds to the promiscuous "colchicine binding site".[12] Indeed, **1** inhibited the binding to tubulin of a commercially available fluorescent colchicine derivative with a similar efficiency as unlabeled colchicine (43% vs 72% at 10 μM), thus, confirming binding to the "colchicine binding site" on its molecular target tubulin.

Several additional compound classes derived from Lavendustin A that inhibit tubulin polymerization have been discovered and reported.[13–15] Moreover, the structure of **1** also resembles another known class of antimitotic agents, the combretastatins[10,16,17] However, the results we obtained for analogs of **1** demonstrate that our substances have to be considered as a new compound class with clear-cut different SARs.

In summary, the most important structural requirement in ring A for high antiproliferative potency of **1** and analogs is a 2,5 or 2,6 substitution pattern with a preference for 2,5-disubstituted analogs. Highest activity is achieved when at least one of the two substituents is the methoxy group. Structural modifications at position 5 have a more pronounced negative effect on antiproliferative activity relative to the same changes at position 2, whereby the activity is generally more sensitive to steric rather than electronic effects of the A-ring substituents. All potent compounds share the same antimitotic mode of action. The most potent representatives

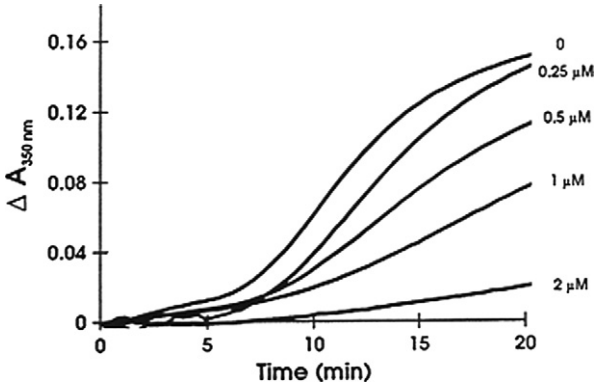


Figure 2. Dose-dependent effect of compound **1** on tubulin polymerization in vitro.

are the 2,5-dimethoxy derivative **1** and the 2-acetyl-5-methoxy analog **5t** with IC_{50} values of 47 and 41 nM, respectively. Based on these results, the 2,5-dimethoxy substitution pattern has been used throughout the subsequent SAR studies, that is, derivation of the spacer and ring B in this compound class. We demonstrated that compound **1** binds to tubulin at the promiscuous "colchicine binding site" and, consequently, inhibits tubulin polymerization. This interference with the spindle apparatus results in inhibition of cell proliferation by blocking the cell cycle in mitosis. The compound class is of high clinical interest since it was demonstrated for compound **1** that it affects interphase microtubules only at much higher concentrations and that it exhibits selective antiproliferative activity against certain tumor cells including multidrug resistance phenotypes without suppressing hematopoiesis.[3]

4. Experimental

4.1. Chemistry

4.1.1. Materials and methods

1,4-Diethylbenzene and 2,4-dimethoxybenzaldehyde were purchased from Fluka AG. 2-Methoxy-, 2,3-dimethoxy-, 3,4-(methylenedioxy)-, 2,5-diethoxy-, 3,4,5-trimethoxybenzyl alcohol, 3-methoxy-, 2-methoxy-5-nitrobenzyl bromide, and 2-*t*-butyl-5-methylanisol, were purchased from Sigma–Aldrich. 2,6-Dimethoxybenzyl alcohol and 2,3,6-trimethoxybenzaldehyde were purchased from Apin Chemicals and Oakwood Products, Inc., respectively. 2-Bromo-5-methoxybenzaldehyde was purchased from ABCR GmbH and 5-chloro-2-methoxybenzaldehyde and 5-formyl-2-hydroxybenzoic acid methylester from 3B Scientific Corporation. 2-Methoxy-5-methylthio-,[18] 2-dimethylamino-5-methoxybenzaldehyde,[19] and 2-acetoxy-5-(bromomethyl)benzoic acid methylester[20] were prepared according to published procedures.

In general, benzyltriphenylphosphonium bromides **3** were prepared by heating an equimolar solution of either the corresponding benzyl bromides and PPh_3 or the corresponding benzyl alcohols and $PPh_3 \times HBr$ (Sigma–Aldrich) in toluene at 80 °C for about 5 h. The solution was cooled, and the solid precipitate was filtered, dried, and used directly without further purification. Some benzyl alcohols were synthesized from the corresponding benzaldehydes by treatment with sodium borohydride in methanol.

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. ^{1}H NMR spectra were recorded at 250 MHz (Bruker WM 250) usually in $CDCl_3$ with $(CH_3)_4Si$ as internal standard. Chemical shifts are given as δ units.

4.1.2. General procedure for the synthesis of test compounds 5 and 6

4.1.2.1. Method A. Wittig reaction + hydrogenation. Synthesis of 5-[2-(3,4,5-trimethoxyphenyl)ethyl]-2-hydroxybenzoic acid methylester (6b). *(E)- and (Z)-5-[2-(3,4,5-Trimethoxyphenyl)ethenyl]-2-hydroxybenzoic acid methylester.* At −30 °C, *n*-butyllithium (0.57 mL, 5.7 mmol, 10 M solution in hexane, Aldrich Chemical Co.) was added to a solution of diisopropylamine (0.57 g, 5.7 mmol) in dry tetrahydrofuran (25 mL). After stirring for 30 min, the mixture was cooled to −50 °C, and phosphonium bromide **3l**[21] (1 g, 1.9 mmol) was added as solid. The orange suspension was stirred for 1 h at −50 °C, cooled to −70 °C, and treated with a solution of **4a** (345 mg, 1.9 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 was poured into aqueous ammonium chloride solution. Extraction with ethyl acetate yielded a product mixture, which was chromatographed on silica gel (hexane/ethyl acetate = 9:1) to give pure (*Z*)-5-[2-(3,4,5-trimethoxyphenyl)ethenyl]-2-hydroxybenzoic acid methylester [168 mg, 26%; colorless crystals: mp 103 °C, NMR δ 10.74 (s, 1H), 7.81 (d, J = 2.2 Hz, 1H), 7.42 (dd, J = .2 + 8.6 Hz, 1H), 6.86 (d, J = 8.6 Hz, 1H), 6.50 (s, 2H), 6.48 (s, 2H), 3.92 (s, 3H), 3.85 (s, 3H), 3.70 (s, 6H)], followed by a mixed fraction (125 mg, 19%) and its pure *E*-isomer [157 mg, 24%; colorless crystals: mp 155 °C, NMR δ 10.78 (s, 1H), 7.97 (d, J = 2.3 Hz, 1H), 7.66 (dd, J = 2.3 + 8.7 Hz, 1H), 7.00 (d, J = 8.7 Hz, 1H), 6.97 (d, J = 16.5 Hz, 1H), 6.91 (d, J = 16.5 Hz, 1H), 6.73 (s, 2H), 3.99 (s, 3H), 3.92 (s, 6H), 3.88 (s, 3H)].

Only 1.2 equiv of lithium diisopropylamide (preferably prepared from 1.6 M solution of *n*-butyllithium in hexane) was used in Wittig reactions with phosphonium bromide **3n** and benzaldehydes **4b–e** as starting materials. In most cases, the stilbene isomers were not separated, but isolated and used as mixture for the next step.

5-[2-(3,4,5-Trimethoxyphenyl)ethyl]-2-hydroxybenzoic acid methylester (***6b***). (*Z*)- or (*E*)-5-[2-(3,4,5-Trimethoxyphenyl)ethenyl]-2-hydroxybenzoic acid methylester or a mixture thereof (160 mg, 0.46 mmol) was dissolved in ethyl acetate (20 mL) and hydrogenated over palladium (20 mg, 10% on charcoal) for 4 h at atmospheric pressure. The mixture was filtered over Celite, and the filtrate was concentrated in vacuo. The residue was crystallized from ethanol or chromatographed on silica gel to afford **6b** (154 mg, 96%) as colorless crystals: mp 90 °C.

4.1.2.2. Method B: O-acetylation. Synthesis of 5-[2-(2,5-diacetoxyphenyl)ethyl]-2-hydroxybenzoic acid methylester (5j). A solution of **5i** (200 mg, 0.7 mmol) in dichloromethane/tetrahydrofurane (2:1) was treated successively with triethylamine (280 mg, 2.8 mmol) and acetic anhydride (230 mg, 2.2 mmol). The mixture was stirred for 2 h at room temperature, poured into water, and stirred for an additional 30 min. Extraction with ethyl acetate gave after drying and evaporation of the solvent a crude product, which was purified by chromatography (silica gel, hexane/ethyl acetate = 4:1) yielding **5j** (102 mg, 39%) as colorless oil.

4.1.2.3. Method C: O-alkylation. Synthesis of 5-[2-(2-ethoxy-5-methoxyphenyl)ethyl]-2-hydroxybenzoic acid methylester (5k). A mixture of **5g** (100 mg, 0.33 mmol), potassium carbonate (50 mg, 0.36 mmol), iodoethane (52 mg, 0.33 mmol), and acetone (7 mL) was heated to reflux overnight. Then it was poured into water and extracted with ethyl acetate. The combined organic layers were dried over magnesium sulfate and concentrated in vacuo. The residue was chromatographed on silica gel (hexane/ethyl acetate = 5:1) to separate the title compound **5k** (39 mg, 36%; colorless oil) and recovered starting material **5g** (46 mg).

4.1.2.4. Method D: C-acetylation. Synthesis of 5-[2-(5-acetyl-2-methoxyphenyl)ethyl]-2-hydroxybenzoic acid methylester (5s). Acetyl chloride (180 mg, 2.3 mmol) was added without cooling to a stirred mixture of **5a** (265 mg, 0.93 mmol), aluminum chloride (370 mg, 2.8 mmol), and dichloroethane (10 mL). The mixture was stirred for 3 h at ambient temperature, then poured into 1 N aqueous hydrochloric acid, and extracted with ethyl acetate. The combined organic extracts were washed with brine, dried over magnesium sulfate, and concentrated in vacuo. The residue was chromatographed on silica gel (toluene/ethyl acetate = 93:7) to obtain pure **5s** (198 mg, 65 %) as colorless crystals, mp 107–108 °C.

Starting with **5b** the same procedure afforded an inseparable *o,p*-isomeric mixture of 2-acetyl-5-methoxy-(**5t**) and 4-acetyl-3-methoxyphenyl)ethyl]-2-hydroxybenzoic acid methylester. Pure **5t** was obtained using the following procedure.

4.1.2.5. Synthesis of 5-[2-(2-acetyl-5-methoxyphenyl)ethyl]-2-hydroxybenzoic acid methylester (5t) and 5-[2-(2-acetyl-4-t-butyl-5-methoxyphenyl)ethyl]-2-hydroxybenzoic acid methylester (6c). Aluminum chloride (1.8 g, 13.6 mmol) was added in one portion without cooling to a solution of **5z** (1.16 g, 3.4 mmol) and acetyl chloride (588 mg, 7.5 mmol) in dichloroethane (40 mL), which resulted in a raise of the temperature of the mixture to 35 °C. This temperature was maintained for about 1 h using a water bath. The mixture was then poured into 2 N aqueous hydrochloric acid and extracted with ethyl acetate. The combined organic extracts were washed with brine, dried over magnesium sulfate, and concentrated in vacuo. The residue was chromatographed on silica gel (cyclohexane/ethyl acetate = 4:1) to obtain a small fraction consisting of **6c** (66 mg, mp 114–117 °C), followed by pure **5t** (683 mg, 61 %) as colorless crystals, mp 74–75 °C.

4.1.2.6. Method E: Hydrogenation. Synthesis of 5-[2-(2-ethyl-5-methoxyphenyl)ethyl]-2-hydroxybenzoic acid methylester (5u). A solution of **5t** (105 mg, 0.31 mmol) in dry ethyl acetate (10 mL) was hydrogenated over palladium (20 mg, 10% on charcoal) for 3 h at atmospheric pressure. The mixture was filtered over Celite, and the filtrate was concentrated in vacuo. The residue was chromatographed on silica gel to afford **5u** (85 mg, 85%) as colorless oil.

4.1.2.7. Method F: Synthesis of 5-[2-(5-dimethylamino-2-methoxyphenyl)ethyl]-2-hydroxybenzoic acid methylester (5r). A mixture of **5y** (103 mg, 0.34 mmol; obtained by standard hydrogenation of the stilbene resulting from reacting 3 h and **4a**), 1 N aqueous sodium dihydrogenphosphite solution (3.4 ml, 3.4 mmol), formaldehyde (0.3 ml, 37% solution in water), and dioxane (12 ml) was heated to reflux for 2 h. Then the mixture was poured into water and extracted with ethyl acetate. The combined organic layers were washed with brine, dried over magnesium sulfate, and concentrated in vacuo. The residue was chromatographed on silica gel (toluene/ethyl acetate = 95:5) to give pure **5r** (59 mg, 53%) as yellowish oil.

4.1.3. Starting materials

4.1.3.1. General procedure for the synthesis of benzyltriphenylphosphonium bromides 3. *4.1.3.1.1. (2,5-Diethylbenzyl)triphenylphosphonium bromide (3i). 2,5-Diethylbenzaldehyde.* A mixture of 1,4-diethylbenzene (1 g, 7.45 mmol), hexamethylenetetramine (1.05 g, 7.45 mmol), and trifluoroacetic acid (10 mL) was heated to reflux overnight. The solvent was distilled off, and the residue was treated with cold water (40 mL). The mixture was basified with sodium carbonate, stirred well for 20 min, and then extracted with ethyl acetate. The combined organic layers were dried over magnesium sulfate and concentrated in vacuo. The residue was chromatographed on silica gel (hexane/ethyl acetate = 10:1) to give the pure aldehyde (651 mg, 54%) as an oil: NMR δ 10.26 (s, 1H), 7.67 (d, J = 2 Hz, 1H), 7.36 (dd, J = 2 + 7.8 Hz, 1H), 7.22 (d, J = 7.8 Hz, 1H), 3.04 (qua, J = 7.5 Hz, 2H), 2.70 (qua, J = 7.6 Hz, 2H), 1.21–1.31 (m, 6H). *2,5-Diethylbenzyl alcohol.* 2,5-Diethylbenzaldehyde (632 mg, 3.9 mmol) was dissolved in methanol (15 mL), and the solution was treated with sodium borohydride (147 mg, 3.9 mmol). The mixture was stirred for 15 min at room temperature and then poured into brine. Extraction with ethyl acetate afforded after drying over magnesium sulfate and evaporation in vacuo the crude benzyl alcohol (630 mg), which was used in the next step without purification: NMR δ 7.09–7.24 (m, 3H), 4.73 (s, 2H), 2.70 (qua, J = 7.5 Hz, 2H), 2.64 (qua, J = 7.5 Hz, 2H), 1,60 (br. s, 1H), 1.24 (t, J = 7.5 Hz, 6H). *2,5-Diethylbenzyl bromide.* Bromine

(583 mg, 3.65 mmol) was added to a solution of 1,2-bis(diphenylphosphino)ethane (727 mg, 1.82 mmol) in dry methylene chloride at 0 °C under argon atmosphere. The mixture was stirred for 30 min, treated with a solution crude 2,5-diethylbenzyl alcohol (600 mg, 3.65 mmol), and then stirred for an additional 30 min. The solvent was evaporated in vacuo, and the residue was well triturated with hexane/diethyl ether (1:1). The mixture was filtered to remove the crystalline phosphinoxide by-products, and the filtrate was evaporated to obtain the crude benzyl bromide (760 mg, 92%), which was used in the next step without further purification: NMR δ 7.09–7.20 (m, 3H), 4.55 (s, 2H), 2.76 (qua, J = 7.5 Hz, 2H), 2.62 (qua, J = 7.5 Hz, 2H), 1.30 (t, J = 7.5 Hz, 3H), 1.24 (t, J = 7.5 Hz, 3H). *(2,5-Diethylbenzyl)triphenylphosphonium bromide (**3i**).* A mixture of crude 2,5-diethylbenzyl bromide (750 mg, 3.3 mmol), triphenylphosphine (1.3 g, 5 mmol), and toluene (20 mL) was heated to 80 °C for 3 h. After cooling, the colorless crystalline product was filtered and washed with diethyl ether (1.38 g, 85%): mp 287–290 °C.

4.1.3.1.2. (2-Ethyl-6-methoxybenzyl)triphenylphosphonium bromide (3j). Mp 242 °C; *2-Ethyl-6-hydroxybenzoic acid ethylester.* A mixture of 1-(3-ethoxycarbonyl-2-oxopropyl)-pyridinium bromide[9] (3 g, 10.4 mmol), ethyl vinyl ketone (0.9 g, 10.7 mmol), triethylamine (1.08 g, 10.7 mmol), and ethanol (400 mL) was heated to reflux for 15 h. The solvent was partly distilled off, and the residue poured into brine. Extraction with ethyl acetate yielded after drying over magnesium sulfate and evaporation in vacuo an isomeric product mixture, which was separated by chromatography on silica gel (cyclohexane/ethyl acetate = 100:2). The first fraction consisted of the unwanted isomer 4-ethyl-2-hydroxybenzoic acid ethylester [NMR δ 10.81 (s, 1H), 7.75 (d, J = 8.1 Hz, 1H), 6.82 (d, J = 1.7 Hz, 1H), 6.72 (dd, J = 1.7 + 8.1 Hz, 1H), 4.39 (qua, J = 7.1 Hz, 2H), 2.63 (qua, J = 7.6 Hz, 2H), 1.40 (t, J = 7.1 Hz, 3H), 1.23 (t, J = 7.5 Hz, 3H)], followed by pure 2-ethyl-6-hydroxybenzoic acid ethylester (pure fraction: 355 mg, 18%): NMR δ 11.24 (s, 1H), 7.31 (t, J = 8 Hz, 1H), 6.84 (dd, J = 1 + 8 Hz, 1H), 6.74 (dd, J = 1 + 8 Hz, 1H), 4.44 (qua, J = 7.1 Hz, 2H), 2.95 (qua, J = 7.4 Hz, 2H), 1.44 (t, J = 7.1 Hz, 3H9, 1.21 (t, J = 7.4 Hz, 3H). *2-Ethyl-6-methoxybenzoic acid ethylester.* Sodium hydride (37 mg, 1.2 mmol; 80% suspension in mineral oil) was added to a solution of 2-ethyl-6-hydroxybenzoic acid ethylester (190 mg, 1 mmol) in dry DMF (8 mL). The mixture was stirred for 20 min at room temperature, treated with iodomethane (0.5 mL), and stirred for an additional hour. The mixture was poured into water and extracted with ethyl acetate. The organic layers were combined, dried over magnesium sulfate, and concentrated in vacuo to obtain the oily product (200 mg), which was used in the next step without further purification: NMR δ 7.28 (t, J = 8 Hz, 1H), 6.83 (d, J = 7.6 Hz, 1H), 6.76 (d, J = 8.4 Hz, 1H), 4.40 (qua, J = 7.1 Hz, 2H), 3.82 (s, 3H), 2.60 (qua, J = 7.6 Hz, 2H), 1.38 (t, J = 7.1 Hz, 3H), 1.21 (t, J = 7.6 Hz, 3H). *2-Ethyl-6-methoxybenzyl alcohol.* A solution of crude 2-ethyl-6-methoxybenzoic acid ethylester (200 mg, 1 mmol) in dry toluene (15 mL) was cooled to −40 °C and treated slowly with diisobutyl aluminum hydride (3 mL, 3 mmol; 1 M solution in toluene). The cooling bath was removed, and the mixture was stirred for 1 h at room temperature. Then it was poured into brine and extracted with ethyl acetate. The combined organic layers were dried over magnesium sulfate and evaporated in vacuo to give the crude title compound (160 mg), which was used in the next step without further purification.

4.2. Biological experiments

4.2.1. HaCaT keratinocyte proliferation assay

HaCaT cells[22] were cultivated in DMEM (Gibco) containing 5% FCS. 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 days of incubation, the extent of cellular proliferation was measured by a colorimetric assay using sulforhodamine B.[23] The results represent the average of at least two independent measurements.

4.2.2. Measurement of antimitotic activity

Cells were seeded into 8-well Lab-Tek plastic chamber slides (Permanox, Nunc, Vienna, Austria) at a final density of 24000 cells/0.4 ml/well. After 24 h, the medium was replaced with fresh medium containing specified concentrations of the test compound (Table 1), which were usually 2- to 5-fold above the corresponding antiproliferative IC_{50} values. After 20 h of incubation time, the medium was removed from each slide chamber, and 90% acetone was added for 5 min at room temperature to fix the cells. DNA was stained with DAPI in PBS (0.5 μg/ml for 1 h at room temperature). Fluorescence microscopy was performed with a Zeiss IM microscope equipped with epifluorescence optics. Cells arrested in metaphase were scored for each sample and the mitotic index (100% × number of mitotic cells/number of total cells) determined. At least 300 cells were counted at each drug concentration tested.

4.2.3. Tubulin polymerization assay[24]

GTP and colchicine were from Sigma, and podophyllotoxin was from Aldrich. Monosodium glutamate was purchased from United States Biochemical. Microtubule protein was prepared from pig brain by two cycles of temperature-dependent assembly-disassembly[25] and drop-frozen in liquid nitrogen after the final cold centrifugation, and then stored at −80 °C. Purified tubulin was prepared from the microtubule protein by a total of three cycles of temperature-dependent assembly–disassembly in 1 M monosodium glutamate, pH 6.6, with 1 mM GTP[26] with the following modification: monosodium glutamate powder was added to the rapidly thawed microtubule protein solution with stirring at 4 °C (i.e., in the cold-room) to a final concentration of 1 M followed by the addition of GTP to 1 mM. The solution was then incubated at 37 °C for 1 h to start the first warm cycle. After the final cold centrifugation, the purified tubulin was stored in aliquots in liquid nitrogen. Polymerization was followed turbidimetrically at 350 nm in a computer-controlled Cary Model 1E spectrophotometer equipped with electronic temperature controllers. Preincubations (15 min, 30 °C) of compounds (initially dissolved in DMSO) with tubulin were performed in 0.8 M monosodium glutamate (pH 6.6) in the absence of GTP in a 0.48 ml volume, with all concentrations referring to a final reaction volume of 0.5 ml. Following the preincubation, the reaction mixtures were chilled on ice, 20 μl of 10 mM GTP was added, and the samples were transferred to cuvettes held at 0 °C (final DMSO conc. = 4% v/v). Polymerization was initiated by a temperature jump to 30 °C. The IC_{50} was defined as the drug concentration (obtained by linear interpolation between data points at specific drug concentrations) required to inhibit the extent of polymerization by 50% following a 20-min incubation (i.e., following the temperature jump).

4.2.4. Tubulin binding assay[27]

Fluorescein Colchicine (FC) was purchased from Molecular Probes. Purified tubulin (2 μM) was mixed with FC (2 μM) ± test substance (10 μM) at 37 °C (15 min), cooled on ice (5 min), and then passed through small gel filtration columns (BioSpin 30 chromatography columns, BioRad). Tubulin comes through the column with any FC bound. Samples are placed in a black microtiter plate and the fluorescence measured in a fluorescence microtiter plate reader. The degree of binding is determined relative to the control sample without test substance (i.e. only tubulin + FC).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.07.039.

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