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Syntheses, biological evaluation and SAR of ingenol mebutate analogues for treatment of actinic keratosis and non-melanoma skin cancer

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ABSTRACT

Ingenol mebutate is the active ingredient in Picato® a new drug for the treatment of actinic keratosis. A number of derivatives related to ingenol mebutate were prepared by chemical synthesis from ingenol with the purpose of investigating the SAR and potency in assays relating to pro-inflammatory effects (induction of PMN oxidative burst and keratinocyte cytokine release), the potential of cell death induction, as well as the chemical stability. By modifications of the ingenol scaffold several prerequisites for activity were identified. The chemical stability of the compounds could be linked to an acyl migration mechanism. We were able to find analogues of ingenol mebutate with comparable in vitro properties. Some key features for potent and more stable ingenol derivatives have been identified.

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A search for bioactive compounds in plants used in traditional medicine has led to the identification of ingenol mebutate (PEP005, ingenol 3-angelate, **1**, Fig. 1) from *Euphorbia peplus*.[1,2] This compound has been developed as the active ingredient in Picato®, a new drug for field treatment of actinic (solar) keratosis (AK) offering a short treatment schedule (2–3 days), providing effective and sustained clearance of AK lesions with a predictable onset and short duration of local skin responses.[1,3]

Ingenol mebutate (**1**) is a 3-mono-ester of the diterpene ingenol and angelic acid. More than thirty different 3-mono-esters of ingenol have been isolated from the plant genus *Euphorbia*, mainly with non-cyclic unbranched lipophilic aliphatic acids like hexadecanoic, decadienoic, decatrienoic and dodecatetraenoic acid.[4,5] 3-Mono-esters of ingenol display high affinity towards protein kinase C (PKC),[6] a family of related serine/threonine kinases mediating a number of important cellular signal transduction responses.[7] Ingenol mebutate (**1**) is an activator of novel (δ, ε, η, θ) and classical (α, β_I, β_{II}, γ) PKC isoenzymes,[8] The therapeutic effect of ingenol mebutate in the treatment of actinic keratosis is believed to be caused by a dual mechanism of action: (i) induction of aberrant keratinocyte cell death and (ii) induction of a lesion-directed immune response, at least partially mediated by PKC.[9]

In our search for novel analogues of ingenol mebutate (**1**) with improved properties, such as chemical stability, potency in activation of the immune system and therapeutic efficacy, we have investigated the consequences of minor structural changes of **1**. The ingenol scaffold is a very rigid structure with four hydroxyl groups in the southern part of which only the 3-OH is esterified with angelic acid in **1**. We have explored the importance of the 5- and 20-OH groups as well as the geometry of the seven-membered ring. Furthermore, we have prepared transposed esters at O-3, and investigated cyclic ether lactones connecting O-3 and O-4 in order to understand the importance of the ester function in relation to biological activity. The results from these studies led us to focus on aliphatic ester analogues of **1** having intact ingenol scaffolds. Herein we report the preparation, the chemical and biological characterization of these novel ingenol 3-angelate analogues and the identification of compounds having improved properties.

With a few modifications we have adopted a previously reported method[10] for the preparation of the new 3-acyl derivatives of ingenol (Scheme 1). The formation of ingenol 5,20-acetonide (**3**), previously described,[11] can potentially lead to several mono

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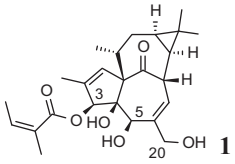


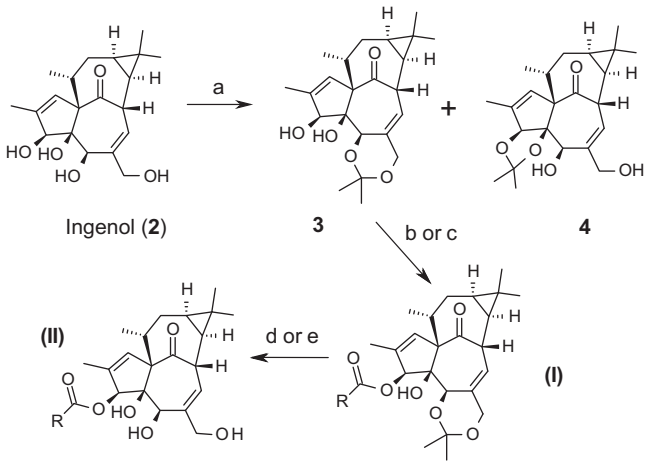
Figure 1. Ingenol mebutate (**1**, PEP005).

acetonide products and a bis-3,4-5,20-acetonide. In our hands, the main product **3** was isolated after crystallization in 73% yield. By chromatographic isolation from the mother liquor, we obtained a minor 3,4-regioisomer (**4**), which was used to prepare some 5-O and 20-*O*-methyl ether analogues of ingenol 3-angelate (cf. Schemes 2 and 3). The angeloylation to form **1** required special conditions to avoid isomerization induced by Michael addition by nucleophilic catalysts.[12] For other 3-acyl-ingenols not associated with this issue, standard procedures, such as Steglich coupling conditions or using acyl chlorides, could be applied in the preparation of **I**. The final acid catalyzed deprotection step to produce **II** was a minor modification of a previously described method.[10,13]

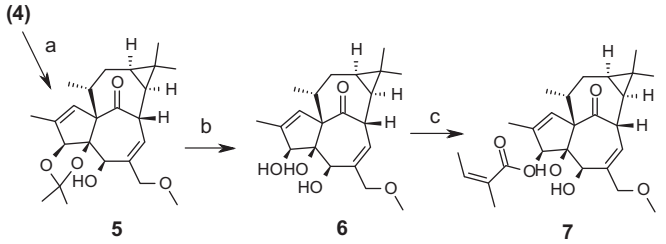
20-*O*-Methyl-ingenol mebutate (**7**) was synthesized from the 3,4-protected ingenol **4** as described in Scheme 2. Treatment of **4** with dimethyl sulfate and lithium bis(trimethylsilyl)amide as base provided **5** in a reasonable yield. Removal of the protecting group followed by angeloylation gave **7**.

The synthesis of 5-*O*-methyl-ingenol mebutate (**11**) was also based on **4** (Scheme 3). The primary alcohol was selectively protected as a triphenylmethyl (Tr) ether. Methylation of **8** followed by removal of the two protecting groups under mild acidic conditions delivered 5-*O*-methyl-ingenol (**9**). The primary alcohol of **9** again required protection prior to esterification which was realized with a *tert*-butyldimethylsilyl ether. Finally, angeloylation of **10** followed by removal of the silyl group under mild conditions provided **11**.

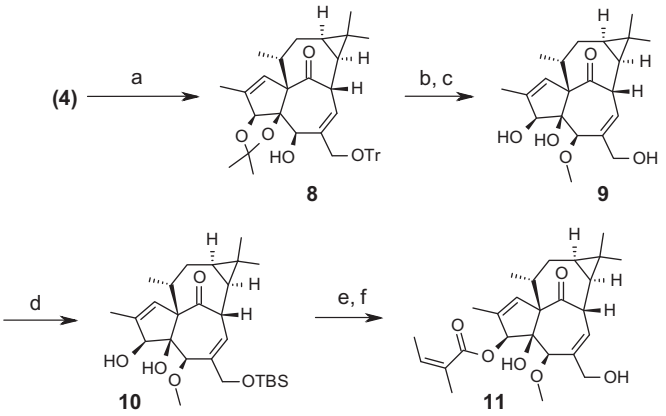
Scheme 4 depicts the preparations of the derivatives **13** and **14** with modified seven-membered rings. The synthesis of the fluoro derivative **13** was inspired by Appendino et al.[14] Selective acetylation of the primary alcohol in ingenol mebutate provided **12**. Treatment of **12** with diethylaminosulfur trifluoride followed by removal of the acetyl group delivered **13**. The two double bonds of ingenol show different reactivities due to the steric hindrance.[11] Thus, the epoxide **14** was prepared by oxidation of **1** with *m*-chloroperoxybenzoic acid and the stereochemistry was assigned from a ^{1}H NOESY spectrum showing the distance between H-7 and H-8 to be about 4 Å in three dimensional space, which is consistent with a trans-configuration.



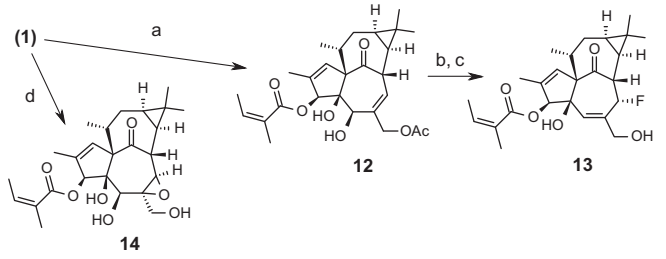
Scheme 1. General synthetic route from ingenol to ingenol 3-acylates (**II**). Reagents and conditions: (a) acetone, PTSA (cat), rt, 0.5 h, 73% for **3**, 4.5% for **4**; (b) RCO_2H, DCC, DMAP, CH_3CN, rt → 140 °C; (c) RCOCl, DMAP, DIPEA, CH_3CN, rt → 140 °C; (d) aq HCl/THF; (e) aq HCl/MeOH, rt.



Scheme 2. Synthesis of 20-*O*-methyl-ingenol mebutate (**7**). Reagents and conditions: (a) $(CH_3O)_2SO_2$ (1.2 equiv), LiHMDS (1.05 equiv), THF, 0 °C → rt, overnight, 35%; (b) aq HCl (4 M), THF, rt, 20 h, 51%; (c) angelic anhydride (1 equiv), Cs_2CO_3 (1.1 equiv), CH_3CN, 0–5 °C, overnight, 47%.

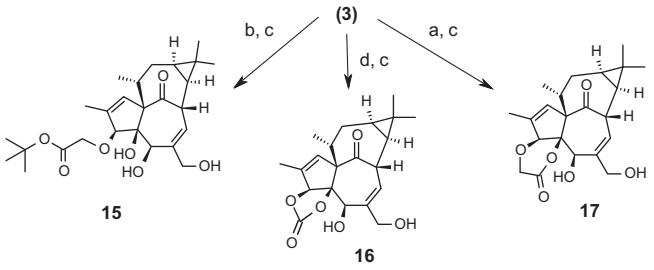


Scheme 3. Synthesis of 5-*O*-methyl-ingenol mebutate (**11**). Reagents and conditions: trityl chloride (1.1 equiv), DMAP, Et_3N, CH_2Cl_2, rt, 62%; (b) $(CH_3O)_2SO_2$ (3 equiv), LiHMDS (2 equiv), THF, −78 °C → rt, 1.2 h; (c) MeOH, concd HCl (cat), rt, 1 h, 7% over two steps; (d) *t*$BuMe_2SiCl$ (2.4 equiv), DMAP (2.9 equiv), CH_2Cl_2, 89%; (e) angelic anhydride (3.7 equiv), Cs_2CO_3 (4 equiv), CH_3CN, rt, 1 h, 70 °C, 1 h; (f) MeOH, concd HCl (cat), rt, 0.5 h, 28% over two steps.



Scheme 4. Syntheses of fluoro analogue **13** and epoxide **14**. Reagents and conditions: (a) Ac_2O (3 equiv), Et_3N, CH_2Cl_2, 88%; (b) DAST (2 equiv), CH_2Cl_2, −78 °C, 2 h; (c) Na_2CO_3, MeOH, rt; (d) MCPBA (4.2 equiv), saturated aq $NaHCO_3$, CH_2Cl_2, rt, 3 h.

The 3-ether **15** and 3-ether-4-lactone **17** modifications were made under the same conditions in two steps (Scheme 5). Alkylation of the 5,20-acetonide **3** with ethyl 2-chloroacetate led to a lactone as main product via a two-step reaction. However, alkylation with the bulky *tert*-butyl ester prevents formation of lactone. Hydrolysis under acidic conditions provided the two 3-ether derivatives **15** and **17**. A cyclic 3,4-carbonate **16** was prepared by reacting **3** with 1,1′-carbonyldiimidazole followed by deprotection as described above.



Scheme 5. Synthesis of compounds modified in the 3-position. Reagents and conditions: (a) ethyl 2-chloroacetate (5 equiv) K_2CO_3 (8 equiv), CH_3CN, 80 °C, 18 h; or (b) *t*-butyl 2-bromoacetate (5 equiv); (c) aq HCl (4 M), THF, rt, 16 h; (d) 1,1′-carbonyldiimidazole (2 equiv), KHMDS (2 equiv), THF, rt, 28%.

To test the biological activity, potencies for the induction of necrotic cell death were determined in HeLa cells, and immune response-related effects were measured as oxidative burst induction, that is, release of reactive oxygen species (ROS), in polymorphonuclear (PMN) leukocytes as well as cytokine release (IL8 and TNFα) in human primary epidermal keratinocytes.[15] Preincubation with Bisindolylmaleimide I,[16] an inhibitor of classical and novel PKC isoforms, abolished ingenol mebutate-mediated cytokine release and PMN oxidative burst, indicating a requirement for PKC activation in these read-outs. Activation of PKCδ was determined for several compounds to confirm the correlation to oxidative burst and cytokine release potency (Table 2).[17] As expected, ingenol (**2**) itself was lacking activity in all assays (cf. Ref. 18).

The 20-*O* methyl ether of ingenol mebutate (**7**) was completely devoid of any oxidative burst and cytokine release activity, which confirms the importance of a free 20-OH group capable to engage as hydrogen bond donor as previously shown for other ingenol 3-acylates with long-chain aliphatic acids.[5,10,14] The 5-O methyl ether of ingenol mebutate (**11**) was almost two orders of magnitude less potent than **1** in the ROS and cytokine release assays. Loss of activity could point towards the need of a free 5-OH group, but the steric influence of the 5-methoxy group on the conformation of the 20-OH group shown to be pivotal for the activity cannot be ruled out. The allylic fluoride, **13**, also lacking a 5-OH, showed approximately 100-fold less cellular activity, which could be a combination of a missing 5-OH and a change of required spatial position of the 20-OH caused by the double bond. No measurable PKCδ activation could be observed for this compound in accordance with the putative involvement of PKC in the cellular test systems. Selective epoxidation of the $\Delta^{6,7}$-double bond of **1** led to compound **14** with one order of magnitude less potency in ROS and cytokine release assays compared to **1**. Thus, the modest modifications of the ingenol scaffold leads to significantly less active compounds (cf. Table 1).

Ingenol is a 1,2,3,5-tetra-ol and this particular alignment makes acyl migration possible.[10] In aqueous solution ingenol mebutate will form an equilibrium between the 3-, 4-, 5- and 20-mono-angelates in an approximate ratio of 1:0:1:18 and with no immediate hydrolysis (Scheme 6). A pH-dependent mechanism for acyl migration in aqueous solution has been proposed for corticosteroid esters,[19] which we believe to be applicable for this system as well. Compounds with improved stability would prolong the shelf-life and facilitate the conditions for distribution of the drug. Ingenol mebutate is most stable at pH 2.0–4.5. In order to quickly rank the stability of new ingenol 3-acylates we determined the recovery at pH 7.4 after 16 h at ambient temperature. Under these conditions 60% ingenol mebutate was recovered. The degradation products of the 3-mono esters of ingenol were identified by LC–MS and they were generally the acyl migration products: 5-mono esters and 20-mono esters. It can be noted that **11** and **13** lacking the 5-OH was stable (>95%) and **7** lacking the 20-OH had a stability (77%) between **1** and **11**, which supports the acyl migration mechanism in the degradation depicted in Scheme 6.

The purpose of making 3-(*t*-butoxycarbonylmethyl) ingenol (**15**) was to produce a derivative not capable of acyl migration, but still containing an ester carbonyl in a neighboring position. The 'transposition' of the ester carbonyl one C-atom away from O-3 of ingenol did produce a stable compound (95% recovered after 16 h) but with greatly reduced ROS and cytokine release activity (Table 1). Likewise, the ether lactone **17** was inactive. The cyclic 3,4-carbonate **16** was roughly 20 times less active than ingenol mebutate (cf. Table 1).[10] Thus the position of the ester carbonyl group as well as the free rotation to allow the carbonyl group to be placed in an optimal position for target interaction is of importance as previously shown in simulations for ingenol 3-benzoate in complex with PKCδ.[20]

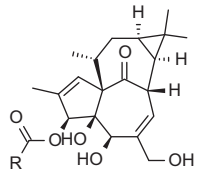
As minor modifications of the ingenol scaffold led to inferior biological activity we started the next phase with exploring the SAR features related to small modifications of the angelate acyl group. We focused on stability as well as ROS and cytokine release potency. Angelic acid is a weak carboxylic acid (pK_a 4.30)[21] and even weaker acids should form more stable esters. However, significantly weaker aliphatic carboxylic acids were not readily

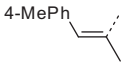
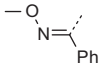
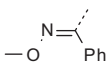
Table 1
Chemical stability and biological activity of 3-mebutate esters of modified ingenol scaffolds and of 3-ether ingenols

No	Stability[a]	Oxidative burst (ROS)[b]		TNFα release[c]		IL8 release[c]		Necrosis[d]
	% Recovered	EC_{50} nM	E_{max} (%)	EC_{50} (nM)	E_{max} (%)	EC_{50} (nM)	E_{max} (%)	LC_{50} (μM)
1	60	8.7	113	11.2	98	10.3	95	230
2	>95	10,000	–	10,000	–	10,000	–	>400
7	77	10,000	–	10,000	–	10,000	–	400
11	>95	281	119	748	131	738	–	Nd
13	>95	1240		1210	67	1130	57	Nd
14	Nd[e]	50	114	367	156	320	119	Nd
15	95	3300	111	10,000	–	10,000	–	311
16	Nd	181	124	253	112	171	109	400
17	25	10,000	–	10,000	–	10,000	–	Nd

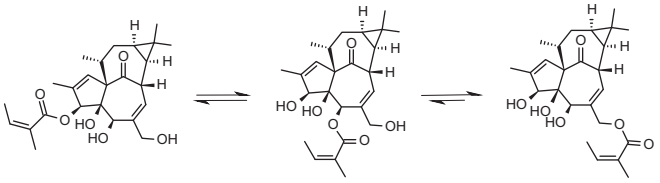
[a] The chemical stability over 16 h was evaluated in an aqueous buffer with less than 30% organic solvent at pH 7.4. Reported as % recovered material.[25]
[b] PMN respiratory burst after 40 min incubation of test compound was quantified by measuring fluorescence expressed in relative light units.[26] EC_{50} denotes the test compound concentration producing 50% of the maximum effect given by **1**. E_{max} indicates the maximal response in relation to **1**.
[c] Cytokine secretions were measured in human adult log-phase primary epidermal keratinocytes after incubation of test compound for 6 h at 37 °C.[27] The EC_{50} and E_{max} calculations according to oxidative burst protocol.
[d] HeLa cells were treated with test compounds for 30 min at 37 °C and then measured the remaining metabolic activity. LC_{50} denotes concentration giving 50% loss of metabolic activity.[28]
[e] Nd: not determined

Table 2
Chemical stability and biological activity of ingenol 3-acylates



No	R-group	Stability[a]	Oxidative burst (ROS)[b]		TNFα release[b]		IL8 release[b]		Necrosis[c]	PKCδ activation[d]
		% Recovered	EC_{50} (nM)	E_{max} (%)	EC_{50} (nM)	E_{max} (%)	EC_{50} (nM)	E_{max} (%)	LC_{50} (μM)	EC_{50} (nM)
1		60	8.7	113	11.2	98	10.3	95	230	4.1
18		0	9.1	110	802	91	821	93	Nd	Nd
19		5	17	112	10,000	–	10,000	–	Nd	Nd
20		55	4.3	109	66	82	86	74	283	Nd
21		20	6.1	114	213	77	246	74	406	Nd
22		30	5.2	102	29	101	21	100	129	Nd
23		45	7.5	92	96	81	112	75	237	Nd
24		75	5.9	106	8.0	97	8.6	102	174	5.3
25		72	7.4	126	4.9	110	3.8	97	103	5.2
26		45	5.9	126	3.1	101	2.1	87	109	Nd
27		18	25	88	318	87	313	115	Nd	Nd
28		30	19	104	112	72	120	63	Nd	Nd
29		60	6.3	109	27	62	43	59	86	4.5
30		30	26	112	243	81	256	83	Nd	Nd
31		10	10	111	450	60	882	55	Nd	Nd
32		30	6.5	107	65	58	69	54	Nd	Nd
33		35	6.0	104	10,000	47	10,000	43	112	Nd
34		60	6.7	99	10,000	42	10,000	45	Nd	Nd
35		30	6.0	110	88	60	182	54	Nd	Nd
36		70	7.6	127	13	62	22	52	61	2.9
37		70	8.3	136	36	55	27	65	77	Nd
38		68	49	120	1600	52	147	54	92	10.6
39		55	28	117	10,000	47	10,000	45	Nd	Nd
40	CH_3-$(CH_2)_8$–	Nd	10	137	11	96	8.0	94	117	5.3
41	CH_3-$(CH_2)_{14}$–	Nd	28	106	8.4	115	7.3	109	400	4.7

[e]Nd: not determined.
[a] Stability was measured according to footnote in Table 1.[25]
[b] ROS, TNFα and IL8 release were measured according to footnotes in Table 1.[26,27]
[c] Necrosis was measured according footnote in Table 1.[28]
[d] Activation potency of PKCδ was measured by the use of an in vitro phosphorylation assay using a PKC-peptide substrate.[29]



Scheme 6. Angeloyl migration in aqueous solution exemplified with **1**.

available, so we focused on steric hindrance as means of controlling acyl migration.[22] A number of aliphatic esters **II** were prepared according to Scheme 1 and tested.[15]

The series of simple α,β-unsaturated 3-acyl ingenol analogues showed that no α-substituent gave poor stability (**18** and **19**) and low potency in cytokine release. The latter result is probably due to significant rearrangement by acyl migration of the test substances during the test conditions (6 h/37 °C/pH 7.4) to the inactive 5- and 20-esters, which had not proceeded during the shorter oxidative burst assay (40 min/37 °C/pH 7.4). Surprisingly, the β,β-disubstituted isomer showed comparable ROS release and stability despite the lack of α-substituent (**20** vs **1**). On the other hand, the α-mono-substituted acrylates **21** and **22** had comparable activity on ROS release but were still clearly inferior to the angelate **1** regarding stability. The ethyl substituted derivative **22** displayed significantly better effect on cytokine release and necrosis than the methyl analogue **21**. The tigloyl stereoisomer **23** showed lower cytokine release potency and was somewhat less stable than the angelate **1**, despite a more favorable pK_a of 5.02[21] (see above). In this particular case, the lower stability of the isomer **23** may well be caused by sterically more favourable conditions for acyl migration. Thus, the alkyl-disubstituted (α,β or β,β) 3-acryl ingenol analogues showed slightly better stability than the monosubstituted compounds, albeit potency in cytokine release was still inferior to the angelate **1**. Of the simple acrylate derivatives only the α,β,β-trimethyl-substituted **24** appeared to be slightly superior to the angelate **1** in all assays performed. Alternatively, a phenyl substituent on the α-position of the unsaturated acyl group provided comparable PKCδ activation but appeared to improve stability and cytokine release potency compared to the level of **1** (**25**), whereas β-substitution only improved cytokine release over **1** (**26**).

Saturated aliphatic acyl groups gave a similar picture. 3-Butanoyl ingenol[10] (**27**) had poor stability and probably as a consequence also poor cytokine release potency. Alpha-substituents in the acyl group (**28** and **29**) increased, as expected, the stability and the latter was equipotent to the angelate **1** in the tests performed, including PKCδ activation (IC_{50} 4.5 nM compared to 4.1 nM for **1**).

A series of three to six-membered cyclic acyl carboxylates (**30**–**35**) all had poor stability, with the exception of the cyclohexenyl **34**, and all compounds were also inferior to the angelate **1** regarding cytokine release properties even if ROS release were comparable to **1**. Only the α-trisubstituted esters **36** and **37** were marginally more stable than ingenol mebutate, and ROS and cytokine release data were comparable to those of **1**, including the PKCδ activation measured for **36**. In addition both **36** and **37** appeared to have a higher cell death induction potential. Acyl groups with oxime functionalities were also investigated (**38** and **39**), without reaching notable potency levels. Thus, both oximes displayed poor cytokine and ROS release and **38** showed moderate PKCδ activation.

From the diversity of structures in Table 2 it appears that potency in the cytokine release and oxidative burst assays do not depend on very strict structural demands on the acid of the ester group. However, it is well known that increased lipophilicity of linear acid side chains enhances the tumor promoting properties of the ingenol 3-acylates,[23] even though the same compound (ingenol 3-hexadecanoate, **41**) may also exhibit anti-tumour activity.[24] Ingenol 3-decanoate and 3-hexadecanoate (**40** and **41**) are potent PKCδ activators and close to equipotent with the 3-angelate **1** in the ROS and cytokine release assays, but these lipophilic compounds are in a special risk zone and might not supply an acceptable safety profile as therapeutic drugs.

The present study shows that the ingenol scaffold conveys several unique structural features, including the 5- and 20-hydroxyl groups, required for the biological activity of the 3-angelate ester **1** linked to induction of oxidative burst, cytokine release and necrosis. However, these essential hydroxyl groups also impact the stability of the ester derivatives as they provide a structural framework for facile acyl migration. The ester moiety positioned in a specific manner in the 3-position was also shown to be essential. We have also provided support, using positive and negative data, for the role of PKC in stimulating the immune response by release of reactive oxygen species (ROS) and cytokines IL8 and TNFα. An extensive SAR exploration showed that a few new compounds were associated with equal or slightly improved overall properties compared to ingenol mebutate. Thus, the α,β,β-trimethyl-substituted **24**, the α-phenyl-substituted **25**, the α-methylcyclohexyl ester **36**, and the noradmantane **37** warrant further investigations on their usefulness in treatment of actinic keratosis and possibly non-melanoma carcinomas (e.g., BCC, SCC and Bowen's disease) as they also display an interesting spread in their ability to induce keratinocyte cell death.

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References and notes

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25. A DMSO stock solution containing a known amount of compound was diluted with pre-heated (37 °C) aqueous buffer pH 7.4 to an organic content ⩽30% v/v. After thorough shaking, the solution was placed in the HPLC auto sampler (37 °C) and injected within 5 min and then repeatedly injected over a period of 16 h. Based on the decrease of area of the compound signal (UV detection at normally 270 nm) the recovery of the compound over time was assessed.
26. Polymorphonuclear leukocytes (PMN) were isolated and purified from fresh buffy coats and incubated for 40 min at ambient temperature with titrated test compound (highest test concentration 10 μM) pre-mixed with hydroethidine. The PMN respiratory burst was quantified by measuring fluorescence expressed in relative light units at 579 nm (excitation: 485 nm) using an Envision plate reader. EC_{50} was calculated as the concentration of test compound producing 50% of the maximum effect given by ingenol 3-angelate (**1**). E_{max} indicates the maximal response in relation to **1**.
27. Human adult log-phase primary primary epidermal keratinocytes were sub-cultured in 96-well plates at 10,000 cells/well and incubated with titrated test compound for 6 h at 37 °C in humidified air/CO_2 (95%/5%). Secreted TNFα levels were quantified using Meso Scale Discovery 4-spot cytokine plates. Secreted IL8 levels were based on homogeneous time-resolved fluorescence (Human IL-8 HTRF® kit, CisBio). EC_{50} and E_{max} were calculated according to previous reference.
28. HeLa cells were treated with increasing concentrations of ingenol esters up to 400 μM for 30 min at 37 °C. Subsequently, metabolic activity was quantified by the use of the resazurin-based dye formulation PrestoBlue (Invitrogen). LC_{50} was calculated as the concentration of test compound producing 50% loss of metabolic activity.
29. PKCδ was tested in the KinaseProfiler™ assay (Millipore) using 0.05 mg/mL phosphatidylserine in the absence of diacylglycerol to allow for observations of any stimulatory effects. The compounds were serially diluted, at semilogarithmic intervals, for twelve points starting from 10 μM. Assays were started by the addition of ATP. Data points for the EC_{50} determinations were performed in duplicate.