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# Synthetic Tigliane Intermediates Engage Thiols to Induce Potent Cell Line Selective Anti-Cancer Activity

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**Abstract:** The tigliane ring system, which encompasses iconic members such as phorbol and TPA, is widely renowned due to numerous observations of displaying potent biological activity, and subsequent use as mainstream biochemical tools. Traditionally, naturally occurring phorboids are regarded as tumor promotors through PKC activation, although in recent times more highly oxidized natural derivatives have been identified as anti-tumor agents. In the view that only limited synthetic investigations toward skeletal stereochemical modification have been undertaken, non-natural systems could be useful for a better understanding of the tigliane pharmacophore via interrogation of cellular sensitivity. In this context the concise construction of a number of highly functionalized non-natural D-ring inverted phorbol esters were synthesized, via a rhodium-catalyzed [4+3] cycloaddition, and biologically evaluated using a range of cancer cell lines. The biological results highlight the notion that subtle changes in structure have dramatic effects on potency. Furthermore, although the non-natural derivatives did not outcompete the natural systems in the PKC-activation sensitive MCF7 cancer cell line, they outperformed in other cancer cell lines (MM96L and CAL27). This observation strongly suggested an alternate mode of action not involving activation of PKC, but instead involves thiol addition as indicated by glutathione addition and NF-κB reporter activity.

Since phorbol (**1**) was first isolated in 1935 from the oil of *Croton tiglium*[1] it has garnered considerable fame as a potent tumor promoter.[2] The closely related naturally occurring esters of phorbol [e.g., 12-*O*-tetradecanoylphorbol-13-acetate, also known as TPA and PMA (**2**)],[3] which are widely deployed biochemical tools for biologists,[4] were initially studied for their tumor promoting activity,[5] but have subsequently been observed to display other profound biological properties (e.g. anti-inflammatory and anti-HIV activity).[6,7] Phorbol esters (e.g. **2**) are protein kinase C (PKC) activators that mimic diacylglycerol (DAG, **3**),[8] but unlike DAG the tigliane diterpenes directly bind to the enzyme.[9] Considerable effort has been invested in mapping the phorbol ester pharmacophore[10] and labeling phorbol esters[11] in an attempt to further understand the structural features of PKC required for optimum binding.[12] Although, PKCs have been traditionally viewed as a family of oncoproteins, more recently they are considered to be a potential therapeutic target,[13] because increasing evidence suggests

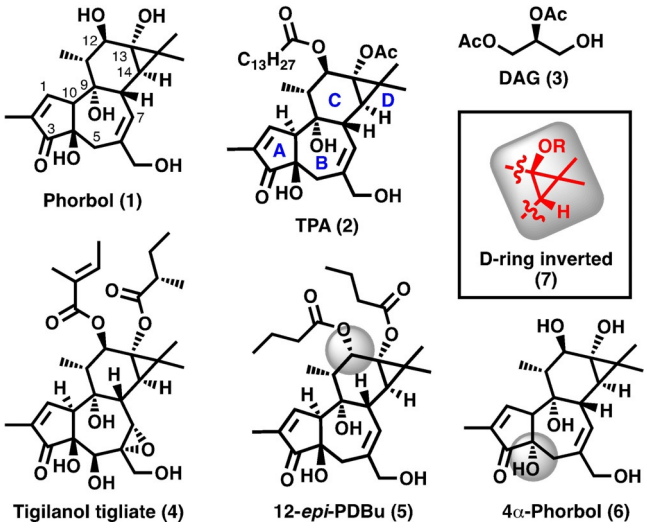


**Figure 1.** Phorbol, related tigliane systems, and the PKC activator DAG (**1**–**7**).

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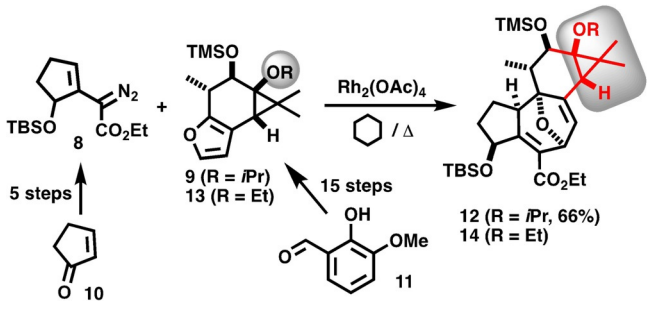
that some PKC isoforms may act as tumor suppressors.[14] In fact, highly oxidized epoxytiglianes [e.g., EBC-46 (**4**)] have been identified as a new class that display considerable anti-tumor activity (Figure 1).[15]

Not only have synthetic chemists been heavily involved in derivatizing the natural tigliane systems (as alluded to above),[16] and in attempting to construct the skeleton itself,[17–19] it is the total syntheses of phorbol[20–22] and related systems (e.g. resiniferatoxin,[23] prostratin,[24] and crotophorbolone[25]) that have required substantial creativity to accomplish. That said, given the amount of synthetic chemistry invested towards (and ultimately achieving) tigliane total synthesis, biological evaluation of fragments *en route* and advanced intermediates has been limited.[26,27] This situation is understandable given it is well known that singular stereochemical changes[28] to the tigliane skeleton (e.g. 12-epi (**5**)[29] and 4α (**6**)[30]) result in much reduced potency. However, little is understood regarding the impact arising from more profound skeletal modifications. Although, based on the above observations (i.e. **5** and **6**), substantial changes away from the natural stereochemistry should in theory give rise to lower potency (Figure 1). In this vein, D-ring inverted phorbol esters (e.g. **7**) were identified as a reasonable starting point to examine this hypothesis for the following reasons: 1) availability of copious *cis*-selective cyclopropanation methods, and 2) potential biological impact on changing tigliane conformation via the smallest ring that is, incremental and pragmatic profound change (Figure 1).

Preliminary synthetic efforts towards the D-ring inversion targets were recently achieved,[31] which provided a platform herein to enable further advancement and derivatization for cancer cell line evaluation.

In brief, synthetic progress was realized via a rhodium catalyzed [4+3] cycloaddition approach,[27,32] involving α-diazo ester **8** and the highly functionalized tetrahydrobenzofuran **9** (Scheme 1). The α-diazo ester (**8**) was accessed in 5 steps from cyclopentanone (**10**),[27] whereas **9** was derived from *o*-vanillin (**11**) in 15 steps.[31] Despite its sensitivity when in contact with either acid or base, the coupling of **9** with **8** gave a single diastereomer of the advanced skeletal framework (i.e., **12**) in 66% yield (Scheme 1).

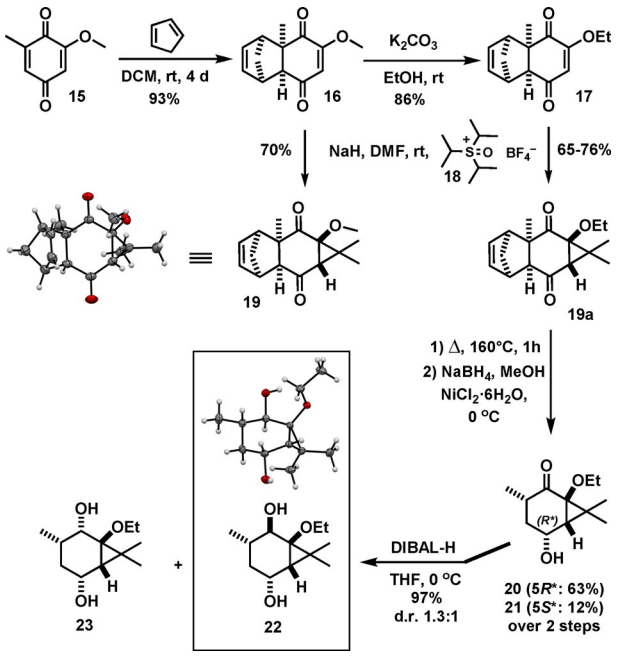
For this study the sequence outlined in Scheme 1 was pursued not only to elaborate the isopropoxy series, but also to explore the ethoxy substituted tetrahydrobenzofuran (of type **13**) in the view that: 1) the less bulky ethoxy group might give rise to other stereoisomers (i.e., by removing the isopropoxy group and reducing reduction selectivity), and 2) the ethoxy group could potentially offer additional degrees of freedom optimum for potency enhancement (i.e. derived from frameworks of type **14**) (Scheme 1).




**Scheme 1.** Construction of the advanced skeletal framework via a rhodium catalyzed [4+3] cycloaddition.

The order of events to access **13** were, however, changed from that reported for the isopropoxy series (i.e. **9**)[31] in that the Diels–Alder reaction with cyclopentadiene was first performed with the methoxyquinone **15** (obtained in 88% yield over two steps from **11**[31]) (Scheme 2). This in part provided overall yield gains, but more importantly opened the possibility of introducing asymmetry using Corey's oxazaborolidine Diels–Alder catalyst (CBS).[33] In this regard it was observed that reaction with (*S*)-CBS gave enantioenriched **16** in 85% yield (87% *ee*), using aluminum tribromide.[34] The use of tin tetrachloride gave the same yield,[35] albeit in slightly higher *ee* (i.e. 90%). However, for rate of progress the remainder of the study was undertaken in the racemic series.

Cyclopropanation to access **19** was performed smoothly with the triisopropyl sulfoxonium salt **18**.[36] However, the methoxy group (i.e. **19**) facilitated undesirable cyclopropyl ring opening in subsequent transformations when using hydride reagents. Fortunately, this problem could be eliminated by substituting the methoxy group for ethoxy, which was achieved using basic ethanol to give **17** in 86% yield. Cyclopropanation was unaffected by this change affording **19a** in yields ranging from 65–76%. Retro Diels–Alder was then immediately followed by employing the known nickel chloride supported

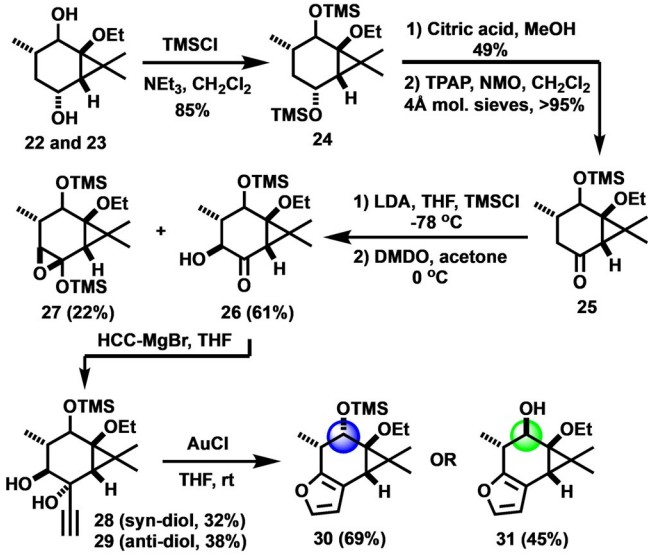



**Scheme 2.** Synthesis of the ethoxy substituted C and D ring system. X-ray crystal structures were obtained for **19** and **22**. Note: when a mixture of **20** and **21** was subjected to DIBAL-H reduction, a second diol diastereomer was isolated, and an X-ray crystal structure was obtained (see **22a** in the Supporting Information).

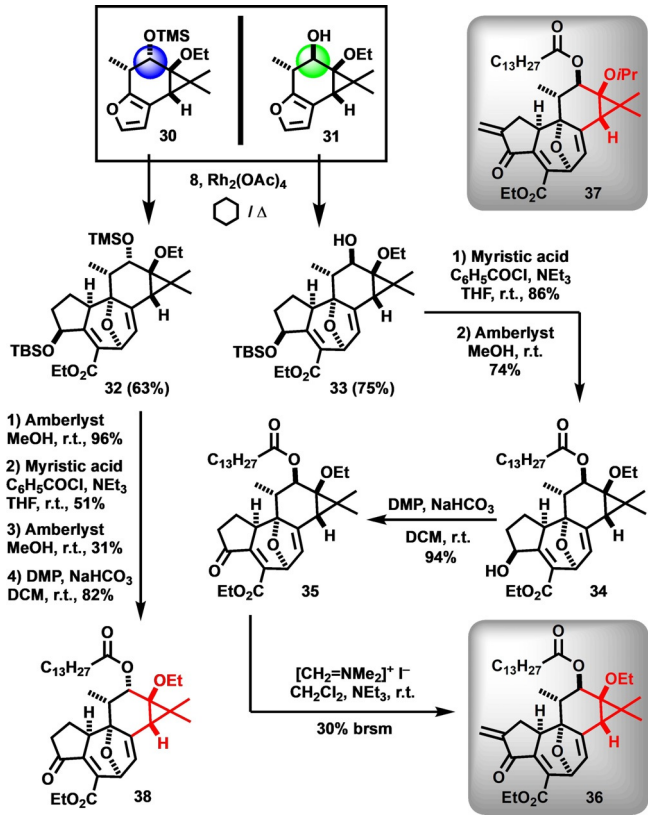
sodium borohydride protocol,[37] which provided regioselective reduction at C5 to afford a major (**20**) and minor (**21**) diastereoisomer. The major diastereomer (**20**) was subjected to diisobutylaluminum hydride reduction affording two diastereoisomers in a combined yield of 97% (Scheme 2).

With **22** and **23** in hand a selective oxidation strategy was pursued. Bis trimethylsilyl protection afforded **24** in high yield (85%), which under controlled conditions was mono-deprotected at C5 using citric acid, and the mono-ol was then immediately oxidized via a Ley-Griffith oxidation (TPAP[38]) to give ketone **25** (Scheme 3). Rubottom oxidation[39] of **25** gave the hydroxy-ketone **26** in 61% yield, along with the intermediate epoxide (**27**) that could be converted into **26** on treatment with mild acid. Addition of ethynyl magnesium bromide afforded the carbinol as a ≈1:1 diastereomeric mixture (i.e. **28** and **29**). Treatment of each individual isomer of the anti-diol (**29**) with gold (I) chloride[40a] interestingly provides protected (**30**) and deprotected (**31**) tetrahydrobenzofurans. The *syn*-diol (**28**) did not produce the desired tetrahydrobenzofuran as expected,[40b] but nevertheless can be recycled by conversion to the anti-diol, via an unoptimized oxidation and reduction sequence.

Both **30** and **31** were amenable to rhodium catalyzed [4+3] cycloaddition with α-diazo ester **8**, affording the individual pentacycles **32** and **33**, in 63 and 75% yields, respectively. Mixed anhydride acylation of the C12 hydroxy with myristic acid, followed by silyl deprotection, gave **34**, which was oxidized with Dess–Martin periodinane. The resulting ketone **35**, isolated in 94% yield, was then converted in one step to the exocyclic enone **36** in 30% yield based on recovered starting material (brsm), using the Eschenmoser salt; according to Crimmins et al[41] (Scheme 4). Interestingly, this method outperformed the Inoue et al. three-step method,[25] which was applied to the isopropyl series (i.e. **37**, 8% over 3 steps). Unfortunately, all attempts to migrate the double bond into the A-ring failed.




**Scheme 3.** Construction of the ethoxy tetrahydrobenzofurans **30** and **31**.




**Scheme 4.** Construction of the advanced skeletal framework via rhodium catalyzed [4+3] cycloaddition, with incorporation of the A-ring exocyclic double bond.

With a robust synthetic route towards D-ring inverted non-natural tigliane esters in place, a structure array was built around the advanced intermediates thus far generated i.e, in the view of assessing potency from a wider perspective. This consisted mostly of the isopropoxy series (i.e. **39**–**46**) with singular inversion of C12 in the ethoxy series (i.e. **38**) (Figure 2). Of the isopropoxy series a number of planned and unexpected synthetic transformations occurred leading to A, B or C-ring modified products. A-Ring changes were the most prevalent and either consisted of increasing (i.e. **40**–**42**) or decreasing (i.e. **43**–**46**) unsaturation. Varying acylation was also a feature (e.g. **44**–**46**) (Figure 2). All of the above modifications took inspiration from biologically active tiglianes[3] and ingenanes.[19]

Advanced tigliane synthetic intermediates **34**–**37** (Scheme 4), and **38**–**46** (Figure 2), were evaluated against melanoma (MM96L), breast (MCF7) and a tongue squamous cell carcinoma (CAL27) cancer cell lines. Select derivatives were tested against leukemia (K562) and hypopharyngeal (FaDu) cancer cell lines, together with human primary fibroblasts (NFF).

For the PKC-activation sensitive cell line MCF7 the natural tigliane esters, TPA (**2**) (Figure 1) and phorbol 12,13-dibutyrate (**47**) (Figure 3), were the most active at $IC_{50}$ values (all in μM) of 0.003 and 0.01 respectively. The most potent of the synthetics were **38** and **35** with $IC_{50}$ values of 3 and 4 respectively, which constituted two to three orders of magnitude lower potency.

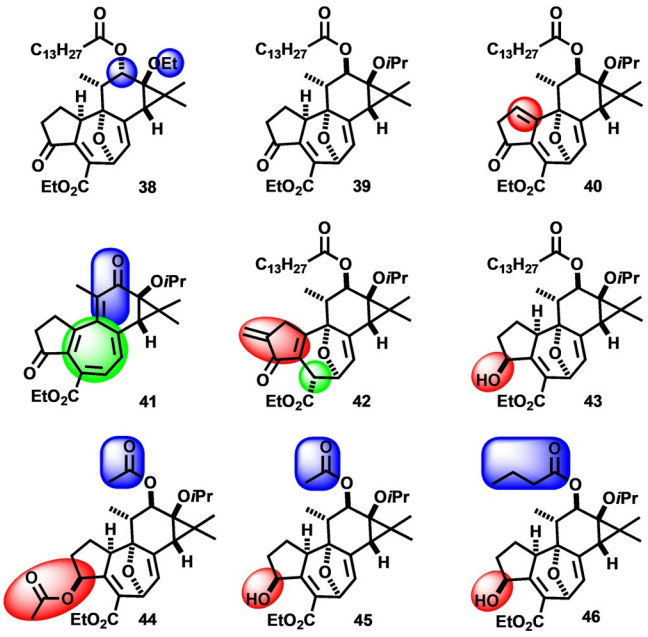



**Figure 2.** An array of modified advanced D-ring skeletal frameworks subjected to cancer cell evaluation. Structural differences, as compared to **39**, are highlighted.

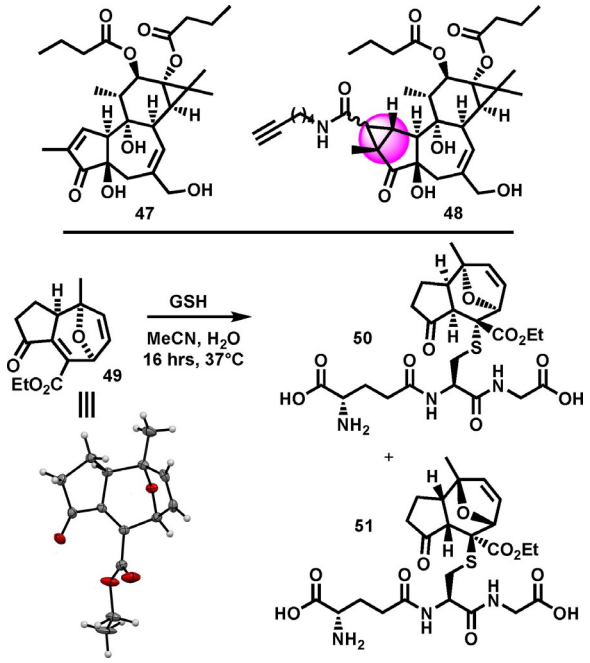



**Figure 3.** Extended derivatives to further understand structure activity relationships and exploration of a Michael addition type mode of action. X-ray structure of **49** is shown.

Intriguingly, however, with the MM96L cell line, **38**, **40**, **39** and **35** were all more active than TPA with $IC_{50}$ values of 1, 4, 5 and 12 respectively i.e., **38** is within error approximately one order of magnitude more potent than TPA. A similarly surprising situation was observed for CAL27 except the observation was more pronounced. For example, five of the advanced synthetic intermediates (e.g. **35**, **39**, **40**, **41**) were more active than TPA, with **38** ($IC_{50}=1$) being approximately 35 times more potent than TPA ($IC_{50}=36$). Of note, the level of potency for TPA is substantially different across cell lines.

In an effort to understand these results further, two additional derivatives were synthesized and evaluated. The first was the only A-ring elaborated phorboid **48** reported to date,[11e] and the second was a simplified A,B-ring system (i.e. **49**) of the advanced synthetic intermediates (Figure 3). In the case of **48** ($IC_{50}=62$) similar activity to that of phorbol 12,13-dibutyrate (**47**) ($IC_{50}=54$) was maintained when tested against MM96L, an observation that was reflected with both CAL27 and FaDu cell lines. However, it differed substantially with MCF7 and K562 in that **48** was two orders of magnitude less potent than **47**. For the truncated system (i.e. **49**) a real surprise developed. This much simplified skeletal core displayed comparable potency to that of the lead synthetic advanced intermediates (e.g. **38** and **39**) with $IC_{50}$ values of 7 for MM96L, and 3 for CAL27, MCF7 and NFF. However, attempts to optimize potency via a small derivative library was not achievable (see supporting information). Based on these, and the above results (see supporting information for full structure activity relationships per cell line), it was clear that the A-ring was sensitive to modification,[42] that is, a ketone is required but exomethylene or any other elaboration/reduction lowered potency. More importantly, however, it seemed that the activated double bond in the B-ring of the synthetics was likely a good Michael acceptor for biochemically relevant cancer cell line processes that is, could there be an alternate apoptosis mechanism not related to PKC activation for MM96L and CAL27 for these systems. Both **38** and **49** were unable to translocate PKCβII to the plasma membrane even at high concentrations (500 μM), further supporting an absent role of PKC isoforms in their activity (see supporting information).

To further interrogate this notion of potential Michael addition (e.g. by thiols) to the B-ring activated double bond,[43] **49** was reacted with glutathione (GSH), and found to form diastereomers **50** and **51** in equal proportion. Inspired by this observation, both **47** and **49** were evaluated as potential inhibitors of NF-κB promoter binding site activation in a HeLa reporter cell line. As NF-κB must be reduced (i.e. disulfide to thiol)[44] in order to bind to its DNA promoter, the ability of **47** and **49** to prevent reporter activity with stimulation by a known activator (500 nM TPA, **2**) was assessed.[45] The results indicate that both **47** and **49** inhibit maximal activation of NF-κB reporter activity induced by TPA, and therefore maybe inhibiting reduction of the transcription factor and subsequent binding to the DNA promoter.

## Conclusions

In conclusion, the study presented herein reinforces previous observations that non-natural changes to the tigliane skeleton can substantially diminish bioactivity against cancer cell lines that are sensitive to activation of PKC isoforms (e.g. MCF7). Surprisingly, however, advanced synthetic intermediates en route to D-ring inverted non-natural tiglianes display higher potency as compared to natural tigliane esters (e.g. MM96L),

especially when moving away from PKC-sensitive cancer cell lines. Structure activity relationships suggest for the first time that: 1) the A-ring is sensitive to modification, 2) the common B-ring double bond is a Michael acceptor via reaction with GSH, and 3) NF-κB reporter activity reinforces the suggestion that thiol addition could be a possible mode of action representing the cancer cell line activity observed herein. Overall, these results provide a foundation for further understanding cancer biochemistry from a synthetic tigliane perspective,[46] and these derivatives may display potential against other thiol-dependent enzymes.[47]

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## Conflict of interest

The authors declare no conflict of interest.

**Keywords:** D-rings · Michael addition · synthetic methods · thiols · tiglianes

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