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Inhibitors of NF-κB Signaling: Design and Synthesis of a Biotinylated Isopanepoxydone Affinity Reagent

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Abstract—A number of inhibitors of NF-κB signaling arising from our recent syntheses of isopanepoxydone and panepoxydone have been identified. Structure–activity data have been correlated to allow the design and synthesis of an affinity reagent for the isolation and identification of any relevant cellular target.

The central role of NF-κB mediated signaling in pro-inflammatory signal transduction has led to intense investigation into the key elements of this pathway.[1] Although multiple enzyme targets are potentially amenable to pharmaceutical intervention, few specific inhibitors have been reported to date, and most agents of clinical utility suffer from concerns regarding their specificity.[2] Recent reports of the potent NF-κB inhibitory activity of the secondary fungal metabolites panepoxydone and cycloepoxydone[3] led to our syntheses of (±)panepoxydone (**2**) and (±)isopanepoxydone (**1**),[4] the latter being a natural congener with a heretofore uninvestigated biological profile (Fig. 1).[5]

Despite tremendous synthetic interest in highly oxygenated α,β-epoxy ketones,[6] relevant specific mode of action data in the form of identified protein/cellular targets are rare. Herein we report an effort designed to identify a biotin-based affinity reagent[7] suitable for the isolation and identification of the panepoxydone binding protein(s).

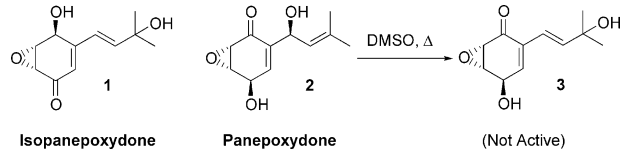
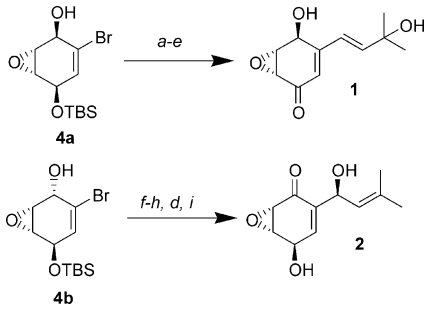


Figure 1. Problematic degradation of panepoxydone.

Early structure–activity studies directed toward identifying a suitable substrate for derivatization revealed that panepoxydone (**2**) undergoes facile allylic rearrangement to a biologically inactive congener (**3**). Although this was of some concern, further investigation (*vide infra*) established that isopanepoxydone (**1**), despite its structural similarity to **3**, possesses a biological profile similar to **2**. This observation, coupled with a number of analogues available directly from the synthetic routes employed to access **1** and **2** (Scheme 1), provided great hope for identifying a suitable biotinylation substrate.[8]



Scheme 1. Total synthesis of isopanepoxydone and panepoxydone. Reagents and conditions: (a) Ac_2O, Et_3N, DMAP; (b) 2-methyl-3-butene-2-ol, $Pd(OAc)_2$, Ag_2CO_3, PPh_3, DMF; (c) TBAF; (d) Dess–Martin; (e) NH_3, MeOH; (f) TBSCl, Imd, DMF; (g) 3-methyl-2-butenal, *t*-BuLi, pentane/ether; (h) NaH, THF; (i) TREAT-HF, MeCN.

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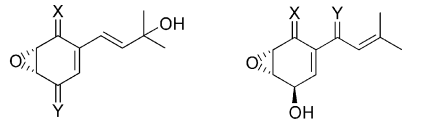
As illustrated in Table 1, the routes used to access **1** and **2** were utilized to produce numerous chemical intermediates bearing strong structural similarities to the active natural products.[9] Assaying these compounds revealed that several non-natural congeners (**7**, **10**, **11**, **13**), in addition to isopanepoxydone, possess NF-κB inhibitory profiles similar to that reported for panepoxydone.[3] In contrast, reduced activity was observed for **5**, **6**, **9**, and **12**; hence, the α,β-epoxyketone moiety appears essential whereas the relative stereochemical configuration about the central core for compounds possessing connectivity analogous to isopanepoxydone appears less important. Within this paradigm, the loss of activity for **8** appears anomalous; however, this analogue decomposes rapidly upon exposure to heat or mild acid.[10]

Table 1. NF-κB inhibitory profile for **1**–**3**, **5**–**13**[9]



1 X = β -OH, Y = O
3 X = O, Y = β -OH
5 X = Y = β -OH
6 X = β -OAc, Y = β -OH
7 X = β -OAc, Y = O
8 X = α -OH, Y = O
9 X = α -OAc, Y = β -OH
10 X = α -OAc, Y = O
13 X = Y = O

2 X = O, Y = β -OH
11 X = O, Y = α -OH
12 X = α -OH, Y = O

Compd	HeLa M Cells	HEK293
Isopanepoxydone (**1**)	≤0	4
Panepoxydone (**2**)	≤0	≤0
3	≥100	63
5	≥100	80
6	≥100	49
7	≤0	≤0
8	≥100	N/D
9	≥100	58
10	≤0	≤0
11	≤0	≤0
12	85	65
13	≤0	≤0
20	≤0	≤0

Whole cell lysates were prepared in 1% NP-40 lysis buffer and clarified by centrifugation at 14,000 *g*. Aliquots (20 μg) were incubated with ^{32}P-radiolabeled NF-κB consensus oligonucleotides (Santa Cruz Biotechnology) and resolved on a native polyacrylamide gel. A phosphorimager was employed to detect autoradiographic images. Densitometric analysis of labeled oligonucleotides retarded by association with liberated NF-κB transcription factors were normalized against samples treated with vehicle or TNFα alone, which established the absolute boundary conditions of 0 and 100.

Additionally, the NF-κB inhibitory profile was found to correlate well with IκBα stabilization (Fig. 2).[3] Thus **7**, **10**, **11**, and **13** attenuated IκBα degradation following stimulation with TNFα, while **3**, **5**, **6**, **9**, and **12** did not (Fig. 2).

Based on our limited structure–activity relationship study, it appeared that sidechain derivatization of an isopanepoxydone analogue would provide the greatest likelihood for success. Rather than attempting biotin attachment to one of the previously prepared analogues at a resident hydroxyl via a labile ester bond, we chose

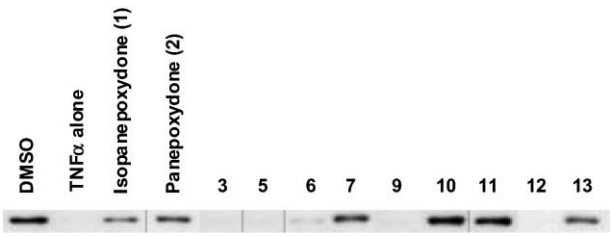
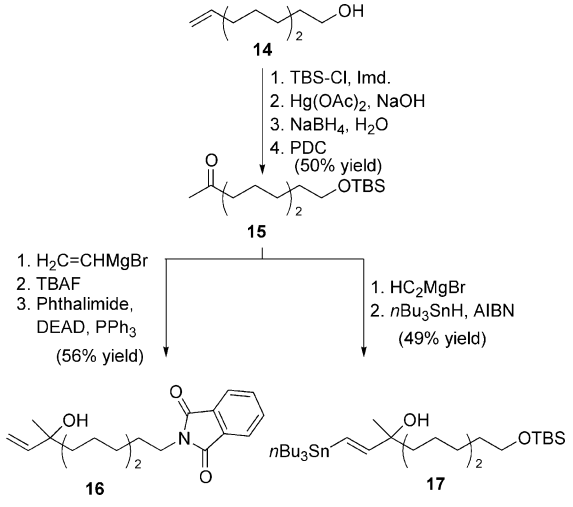


Figure 2. HEK293 cells were treated as described in Table 1. Whole cell lysates were prepared with 1% NP-40 lysis buffer. Following protein concentration normalization, 40 μg were resolved by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane for Western blotting against IκBα using a polyclonal rabbit antibody (Santa Cruz Biotechnology).

to take further advantage of key intermediate **4a**, which is poised for providing access to numerous sidechain analogues via either a Heck or Stille coupling reaction.

Based on these design issues, we targeted an isopanepoxydone analogue possessing a completely modified sidechain wherein a distal latent amine moiety serves as a potential biotinylation site (i.e., **24** *vide infra*). As illustrated in Scheme 2, we began the synthesis of two potentially useful sidechain units from alcohol **14** which was subjected to silylation, oxymercuration–demercuration, and chromium mediated oxidation to furnish ketone **15** (50% overall yield). Subsequent addition of vinylmagnesium bromide, desilylation, and Gabriel amine synthesis furnished Heck substrate **16**. Likewise, a corresponding Stille coupling partner (**17**) was prepared from **15** via addition of ethynyl Grignard and radical mediated hydrostannylation.

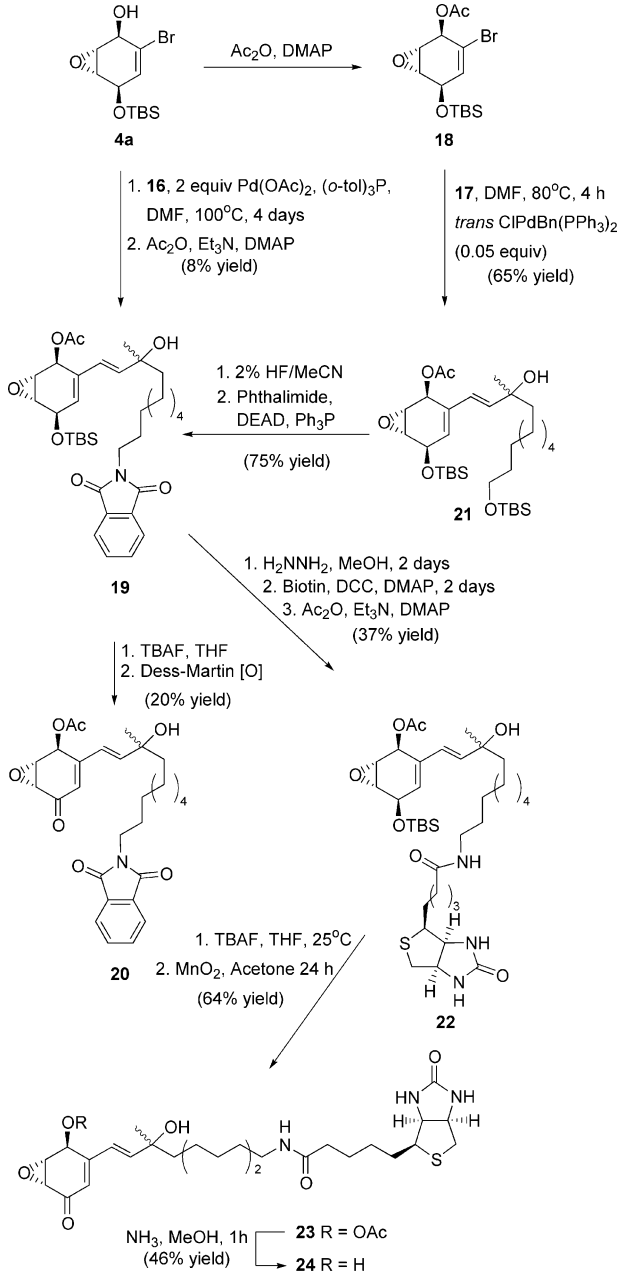
With **16** and **17** in hand, we explored their coupling chemistry (Scheme 3). Initial studies focused on the coupling of **16** with **4a**; however, this substrate combination proved inefficient and efforts to optimize production of **19** beyond 10% failed. Despite the low yield, quantities of **19** sufficient for its conversion to **20** were produced, thus enabling a brief exploration into the effects of sidechain derivatization on biological activity.



Scheme 2. Preparation of sidechain precursors.

We were pleased to find that **20**, utilized as a 1:1 diastereromeric mixture at the tertiary alcohol, demonstrated excellent NF-κB inihibitory properties (Table 1). Encouraged by this result, we turned our efforts toward advancing **20** to a biotinylated analogue. Unfortunately, these efforts were thwarted by the isopanepoxydone core, which proved unable to withstand conditions required for phthalimide deprotection.

Having identified an excellent biotinylation substrate and gained insight with regard to limitations on side-chain introduction and phthalimide deprotection, we decided to focus our attention on a route wherein the Stille substrate (**17**) would serve as the coupling partner and deprotection of the phthalimide would precede completion of the cyclohexene core. Towards this end, we were pleased to find that acylation of **4a** followed by coupling with vinylstannane **17** smoothly furnishes the Stille-coupled bis-TBS ether (**21**) in good overall yield. Selective removal of the primary TBS ether by brief treatment with 2% HF in acetonitrile followed by introduction of the phthalimide moiety under Mitsunobu conditions provided access to **19** with dramatically improved efficiency. In accord with previous observations, we sought to install the required biotin moiety prior to adjustment of the core oxidation state. Thus, **19** was deprotected with hydrazine in MeOH at room temperature and exposed to biotin under standard coupling conditions, acetylation of the derived intermediate then furnished **22**. Desilylation of **22** followed by mild allylic alcohol oxidation gave **23** which, upon deprotection with methanolic ammonia, furnished the targeted affinity reagent (**24**).



Scheme 3. Preparation of the biotinylated analogues.

Turning to the biological investigations, we noted that **24** manifests a much reduced NF-κB inhibitory profile compared to the natural product leads. However, biotinylation of small molecules is often accompanied by an attenuation of activity, and affinity reagents suffering from similar reductions in potency have been successfully employed in the past.[7] Consequently, we investigated the biochemical utility of **24** as an affinity reagent. Treatment of exponentially growing HeLa M cells with 10 μM **24** specifically labels a protein species ca. 50 kDa (Fig. 3) whose interaction with **24** can be competitively titrated by pretreatment with 5-fold molar excess isopanepoxydone (**1**).

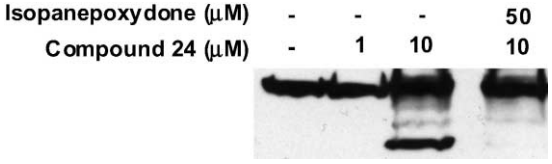


Figure 3. HeLa M cells were pretreated with vehicle or **1** for 1 h prior to treatment with isopanepoxydone biotin (**24**) for an additional h. Whole cell lysates were prepared in 1% NP-40 lysis buffer, normalized for total protein concentration, and resolved by SDS-PAGE. Following transfer to PVDF membrane, biotinylated proteins were identified using a streptavidin–horseradish peroxidase conjugate and visualized with an enhanced chemiluminescence (ECL) reagent (Santa Cruz Biotechnology). The upper nonspecific band is an endogenously biotinylated protein.

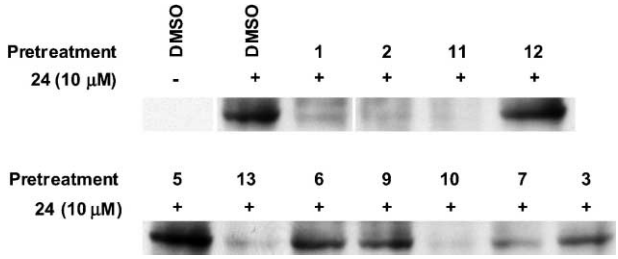


Figure 4. HEK293 cells were pretreated with vehicle, **1**–**3**, **5**–**7**, and **9**–**13** (100 μM) for 1 h prior to challenge with 10 μM **24** for an additional h. Whole cell lysates were prepared, resolved, and visualized as in Figure 3.

We demonstrated the biochemical relevance of this finding by iterative substitution of our panel of isopanepoxydone congeners as pretreatment reagents in our biochemical labeling experiment (Fig. 4). Their ability to compete for binding to our candidate ca. 50 kDa protein correlates well with their biological profiles (Table 1, Fig. 2).

In conclusion, we have prepared a panel of panepoxydone congeners, including the natural product isopanepoxydone, which possess a range of NF-κB inhibitory profiles. Our total synthesis of isopanepoxydone has served as a platform for the preparation of an isopanepoxydone affinity reagent (**24**), which specifically labels a candidate target protein whose characterization will be described in due course.

Acknowledgements

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9. Compounds **5**–**13** were prepared using the same set of reactions illustrated in Scheme 1 from either **4a** or **4b**. From **4a**: (**5**) a, b, K_2CO_3, MeOH, c; (**6**) a–c; (**7**) a–d; From **4b**: (**8**) a–e; (**9**) a–c; (**10**) a–d; (**11**) f, g, h, d, i; (**12**) f, g, d, i; (**13**) a, b, K_2CO_3, MeOH, c, d.

10. The *syn* epimer of isopanepoxydone is extremely labile under thermal (40 °C) or acidic conditions, giving complex mixtures of aromatic byproducts.