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Synthesis of a Simplified Bryostatin C-Ring Analogue That Binds to the CRD2 of Human PKC-α and Construction of a Novel BC-Analogue by an Unusual Julia Olefination Process

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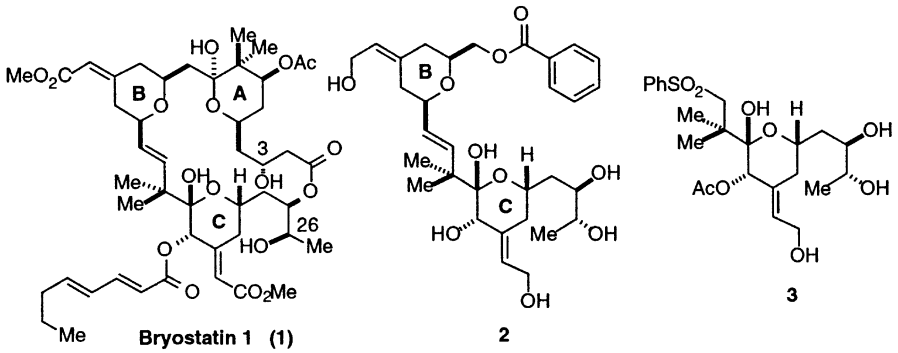
ABSTRACT



The synthesis of two truncated bryostatin analogues 2 and 3 is described. High-field NMR measurements on the C-ring analogue 3 in C^2H_3CN containing 25% ^2H_2O have shown that it binds to the CRD2 of human PKC-α at virtually the same position as phorbol-13-acetate (PA) and bryostatin 1 (1). NMR titration studies have also revealed that 3 binds to the CRD2 with a potency similar in magnitude to PA but much less potently than 1.

Protein kinase C (PKC) isozymes are serine and threonine kinases that play a pivotal role in the signal transduction pathways that control mammalian cell division.[1] They phosphorylate a range of intracellular protein targets[1,2] that drive the processes of mitogenesis,[3] tumorigenesis,[3] growth arrest,[3] and differentiation,[4] and they are activated by various extracellular signals. Not surprisingly, their over- or under-expression in many cells has been correlated with the transition into malignancy[3] and with increased tumorigenicity.[3] For example, in A549 human lung cancer cells, PKC-α levels are considerably higher than in other nearby healthy alveolar tissue.[5] PKC-α levels are also significantly elevated

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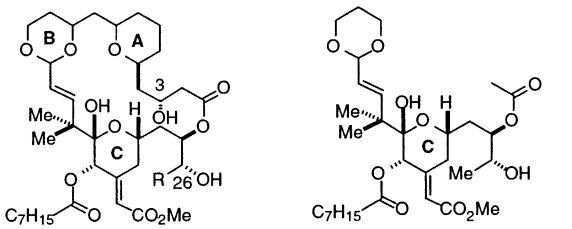
in MCF-7 human breast cancer cells compared with normal breast tissue.[5] In NIH-3T3 cells, PKC-ϵ overexpression causes them to become highly neoplastic and tumorigenic toward nude mice,[4] while PKC-δ overexpression halts proliferation.[4] In mouse keratinocytes, PKC-δ overexpression results in apoptosis,[6] while in rat vascular smooth muscle cells, it causes a dramatic decrease in the levels of cyclins D1 and E. The latter enzymes are critically involved in cell cycle progression.[7] PKC-δ overexpression in this system also up-regulates p27, which is a cyclin-dependent kinase inhibitor.[7] PKC-δ has additionally been implicated in the antiproliferative signaling pathway that is mediated by cell−cell contacts.[8] It will thus be appreciated that PKCs can not only serve as oncogenes in certain circumstances but also function as tumor-suppressors, depending upon their exact levels of expression and their precise distribution within cells.

One naturally occurring antitumor agent that binds selectively to a range of different PKC isoforms, to thereafter modulate their levels of expression, is bryostatin 1 (**1**).[5,9,10] It potently inhibits the growth of several cancer cell lines in a way that correlates closely with its selective down-regulation of PKC-α.[5,9b] It can also halt the growth of some cell systems by apparently preventing certain PKC-δs from undergoing down-regulation.[11]

Bryostatin 1 (**1**) binds to the diacylglycerol/phorbol ester binding sites of phosphorylated PKC isozymes at two highly conserved regions known as the cysteine-rich domains 1 and 2 (PKC CRDs 1 and 2),[12] and in so doing, it creates ligated proteins that have greatly differing intracellular translocation properties,[5] as well as differing susceptibilities toward undergoing degradation[13] (and down-regulation) at their final intracellular destination(s).

Much has still to be learned about how the different PKCs regulate cellular proliferation, differentiation, and morphology, and it is clear that our understanding will only improve if increased access is gained to novel PKC-interactive probe molecules. The fact that bryostatin 1 can selectively modulate PKC isozymes at very low drug concentrations, and simultaneously prevent tumor cell growth, makes it an especially attractive lead for the design of new PKC probes, for there is the added possibility that some of these structures will themselves function as novel anticancer drugs through their effects on deregulated PKC-signaling pathways.

So far, only Wender's group has made substantial progress on the bryostatin analogue/probe front,[14] their team having already identified several simplified structures that combine good PKC inhibiting activity with powerful in vitro antitumor effects (see Figure 1). Their cumulative data on this class



4 R = H PKC K_i = 0.25 nM
5 R = Me PKC K_i = 3.4 nM

6 PKC K_i = >10,000 nM

Figure 1. Some of Wender's bryostatin analogues.

suggest (1) that a 20-membered macrolactone ring, containing an intact "Southern Hemisphere", is needed for good PKC binding activity; (2) that a C(3)-hydroxyl with (*R*)-stereochemistry is important for a high enzyme affinity; and (3) that a free hydroxyl at C(26) is essential for a good interaction with PKC isozymes. Of further relevance is Wender's observation that the B-ring exocyclic olefin and the A-ring can both be deleted from 20-membered macrolactone analogue structures without having serious consequences for PKC-binding affinity.

One of the main goals of our PKC probe/analogue program in the bryostatin area is to characterize the binding of our simplified probe molecules to the CRDs of various human PKCs by NMR methods. The primary advantage of using this technique to identify novel probe structures is that one can readily establish whether a particular analogue/probe is binding to the relevant CRD of a human PKC in exactly the same way as the lead structure (viz. bryostatin 1). The NMR approach also allows small and often quite subtle changes in ligand binding to be easily detected; these can subsequently be correlated with downstream effects on PKC signaling and with the antitumor profile of a particular analogue. The NMR method also gives precise information on which amino acid residues must be targeted in a PKC to produce a particular biological effect, enabling the rational design of new analogues with tailored biological profiles.

At the outset of this project, we thought it important to identify the minimum pharmacophoric elements of bryostatin 1 that would be needed for successful binding to the CRD2 of human PKC-α. Given that Wender and co-workers[14] had already demonstrated that substantial changes could be

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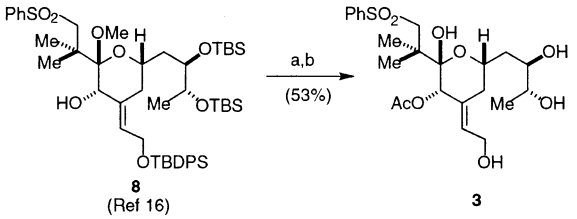
tolerated within the A- and B-regions of their PKC-binding bryostatin analogues, we decided to investigate whether C-ring analogues that lacked both the 20-membered macrolactone ring and the A- and B-ring systems could still bind effectively to the CRDs of human PKCs.

Although Wender's results on the C-ring enal **6** (Figure 1) had not been especially encouraging in this respect,[14] we remained undaunted, for the X-ray crystal structure of tetradecanoyl phorbol-13-acetate **7** (TPA) complexed to the CRD2 of murine PKC-δ had very clearly shown that only two of its hydroxyls, OH(20) and OH(4), and its C(3)-carbonyl were involved in binding of the ligand to the protein.[12] Moreover, the fact that all three of these TPA recognition sites were positioned within a short five-carbon chain,[15] suggested to us that the bryostatin macrolactone system might not actually be necessary for effective binding to PKC and that a truncated C-ring analogue which lacked this feature might still bind to the CRD2, provided its H-bonding capabilities were suitably magnified. However, such a system would still need to project an appropriately oriented lipophilic group from its core to create the long, continuous, hydrophobic protein domain needed for PKC membrane insertion.[15] Herein, we now report on how such planning recently allowed a C-ring analogue **3** to be identified, which binds to the human PKC-α CRD2 with an affinity of the same order of magnitude as phorbol-13-acetate (PA).

Our pathway to **3** set off from the known phenyl sulfone **8**, whose synthesis had previously been described by us from (*E*)-1,4-hexadiene.[16] Compound **8** (Scheme 1) underwent

Scheme 1. Pathway to C-Ring Analogue 3[a]

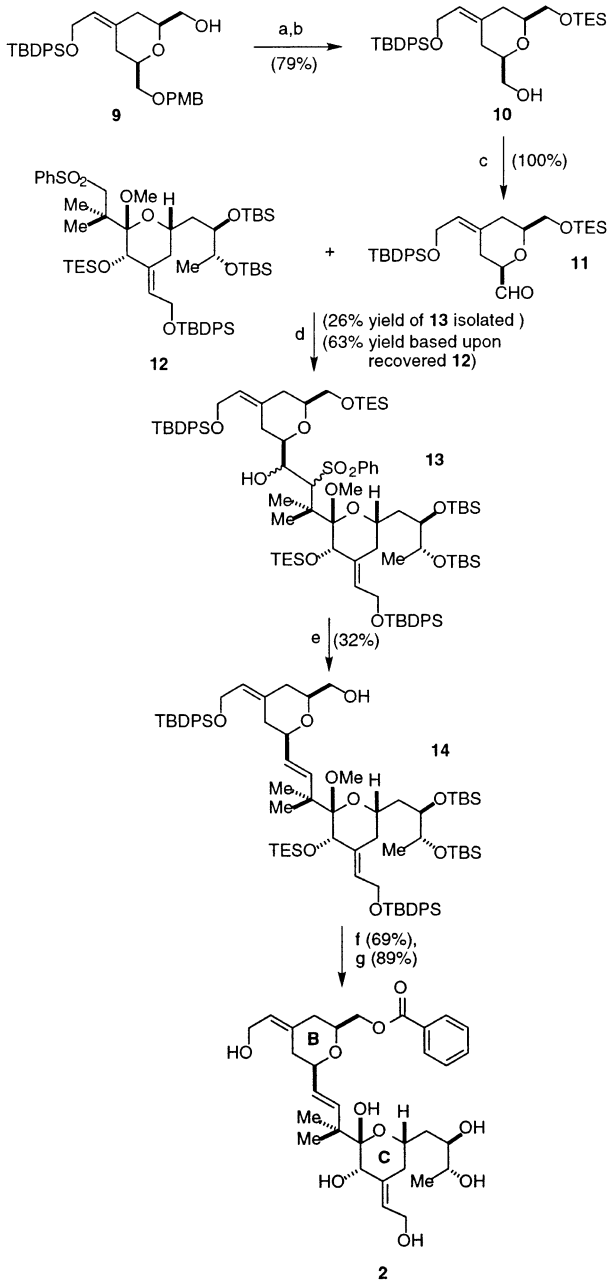


[a] Reagents and conditions: (a) Ac_2O (5 equiv), Py (5 equiv), DMAP (0.5 equiv), CH_2Cl_2 ([**8**] = 0.05 M); (b) 40% aq HF (4 equiv), MeCN/THF (1:1, 0.05 M), 2 h.

smooth acetylation with acetic anhydride, pyridine, and DMAP to give the corresponding *O*-acetate, which readily underwent *O*-desilylation with 40% aq HF in 53% overall yield. Importantly, these conditions also caused glycoside hydrolysis to provide **3** as a 3.6:1 mixture of ring-closed/ring-opened hemiketals. The interaction of **3** with the human PKC-α CRD2 was subsequently studied by 600 MHz ^{1}H NMR spectroscopy.

With the kind assistance of Dr. Peter Parker of the Cancer Research UK Laboratories at Lincoln's Inn Fields, we prepared a CRD2 construct of human PKC-α and verified its sequence and folding by comparison of its ^{1}H spectrum in ^2H_2O with the previously published resonance assignments of Ichikawa[17a] and Hommel.[17b] On addition of PA or phorbol-12,13-dibutyrate (PB) to our CRD2 in ^2H_2O, the ligand−protein complexes immediately precipitated. Following a

Scheme 2. Synthesis of BC-Analogue 2[a]



[a] Reagents and conditions: (a) Et_3SiOTf (1.5 equiv), 2,6-lutidine (5 equiv), CH_2Cl_2 ([**9**] = 0.08 M), −20 °C, 7 min; (b) DDQ (1.5 equiv), CH_2Cl_2/H_2O (18.1), rt, 1.5 h; (c) TPAP (0.1 equiv), NMO (2 equiv), MeCN (0.05M), 4A MS, rt, 0.5 h; (d) **12**, PhLi (1.8 M solution in hexane, 1.26 equiv), THF (0.1 M), −78 °C; warm to −50 °C for 20 min, re-cool to −78 °C, add **11** in THF (0.1 M) dropwise, stir 35 min, warm to rt for 2 h, quench with saturated aqueous NH_4Cl at −78 °C; (e) to **13** in MeOH/EtOAc (2:1) ([**13**] = 0.01 M) at −20 °C was added 6% Na/Hg (2.5 g per 80 mg of **13**) and Na_2HPO_4 (1.25 g per 80 mg of **13**) over 4.5 h, then warmed to 0 °C for 2 h; (f) BzCl (1 equiv), DMAP (0.1 equiv), Py (0.23M), CH_2Cl_2 ([**14**] = 0.015 M); (g) 40% aq HF (6 equiv), THF/MeCN (2:1, 0.02 M), 0 °C, 0.75 h, rt, 12 h.

suggestion in the literature,[18] we added 25% C^2H_3CN to these complexes, which markedly improved their solubility. A detailed comparison of the 1D and 2D spectra of the CRD2 in this solvent system confirmed that the structure of the protein had not been significantly affected,[19] indicating that it will be a generally useful solvent mixture for studying the binding of very hydrophobic ligands to this protein.

A detailed description of the ^1H resonance assignments for the PKC-α CRD2 in 25% aq C^2H_3CN, and of the effects of ligand binding, will be presented elsewhere.[19] Residues specifically affected by binding of PA and PB correspond to those observed in the binding pocket of the TPA−PKCδ CRD2 X-ray crystal structure.[12] Bryostatin 1 also affects several of these residues but clearly binds much more tightly than PA or PB. Our observations conclusively demonstrate *for the first time ever* that the binding site of bryostatin 1 does indeed overlap with that of PA and PB. The binding of our simplified analogue **3** also affected some, but not all, of the residues that bind TPA, indicating that **3** occupies a part of the same binding pocket. Chemical shift changes in the amide region were observed over similar concentration ranges for **3** and for PA and PB, showing that their binding constants are very similar under our conditions. Our combined data thus indicates that it is possible to design bryostatin C-ring analogues that bind specifically to the human PKC-α CRD2, in a novel manner, and with an affinity of the same order of magnitude as those of the phorbol esters.

As the prelude to future protein NMR binding studies with structurally more elaborate BC-fragments, we have recently completed a synthesis of the novel BC-analogue **2**. Importantly, our synthesis has highlighted a number of potential problems that can be faced when using the Julia olefination reaction to connect B- and C-ring bryostatin fragments together.

Our route to **2** (Scheme 2) converted our previously reported alcohol **9**[20] into the aldehyde **11** in three steps. Considerable experimentation was required before conditions were identified that could successfully metalate the phenyl sulfone **12**. *n*-BuLi, *t*-BuLi, MeLi, and LDA all proved unsatisfactory in this regard. However, by adopting the PhLi/THF metalation conditions of Masamune,[21] we were able to eventually prepare the desired anion and add it onto aldehyde **11** to obtain **13** in 26% isolated yield. However, the actual yield of **13** was closer to 63% when it was calculated upon the quantity of recovered **12**.

Unfortunately, difficulties were also encountered when we attempted to *O*-acylate the intermediary *β*-lithioalkoxy- and *β*-hydroxyphenyl sulfone adducts of this union with various acylating agents (e.g., BzCl, Ac_2O, MsCl, etc.). Desulfonylative elimination was therefore attempted directly on **13** using 6% Na/Hg amalgam. To our delight, the partially deprotected alkene **14** was obtained as a single geometrical isomer, but only in 32% yield. To complete the synthesis of **2**, alcohol **14** was *O*-benzoylated and the product globally deprotected using 40% aq HF in MeCN.

In summary, we have shown that it is possible to design a bryostatin C-ring analogue that can bind to the CRD2 of human PKC-α with an affinity of the same order of magnitude as phorbol-13-acetate. Importantly, we have shown that a 20-membered macrolactone structure is not a prerequisite for effective analogue binding to the PKC-α CRD2. Future work will focus on elucidating the structure of the complex formed between **3** and the $^{13}C/^{15}N$-labeled PKC-α CRD2 by high-field NMR. NMR binding studies will also be conducted with **2** and the CRD2, and analogues **2** and **3** will have their antitumor and PKC-modulating properties fully evaluated.

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Supporting Information Available: 500 MHz ^1H and 125 MHz ^{13}C NMR and mass spectra of all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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