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INCREASING THE CELLULAR PKC INHIBITORY ACTIVITY OF BALANOL: A STUDY OF ESTER ANALOGS

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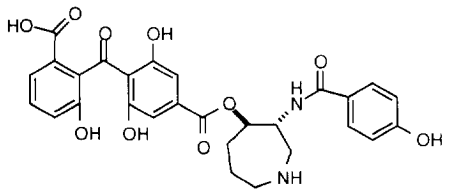
Abstract: As part of an effort to enhance the cellular activity of balanol analogs **2** and **3**, we prepared a series of benzophenone ester analogs of varying steric size and hydrolytic stability. These were evaluated for protein kinase C inhibitory activity, with the results showing that both small alkyl esters and the pivaloyloxymethyl ester analogs displayed large increases in cellular PKC inhibition.

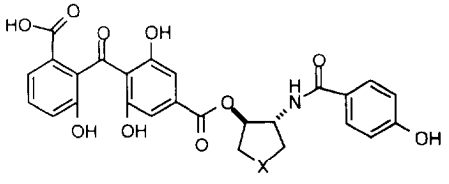
Balanol ((-)-**1**), an unusual metabolite produced by the fungus *Verticillium balanoides*, was recently isolated and found to be a potent inhibitor of protein kinase C with activity in low nanomolar concentrations.[1] Protein kinase C (PKC) plays a key role in cell growth, metabolism and differentiation and is therefore implicated in several diseases involving these processes.[2] Our study of balanol and related analogs sought to develop a novel balanol analog with therapeutic utility.

Studies on structure / activity relationships revealed the importance of the carboxylic acid group for PKC inhibitory activity.[3] However, it was thought that the hydrophilic nature of this group contributed to the lack of activity displayed by balanol in cellular assays. Therefore, a series of acid replacements, namely esters, were synthesized in an attempt to enhance the bioavailability of the compounds by increasing their hydrophobicity.

Early in our balanol investigations two azepine replacements, a pyrrolidine ring and cyclopentane ring, were found to give analogs (**2** and **3**) which displayed comparable PKC inhibitory activity to balanol.[4] These ring systems were used in the ester series reported here for their ease of synthesis over the azepine ring.



(-)**1**, balanol

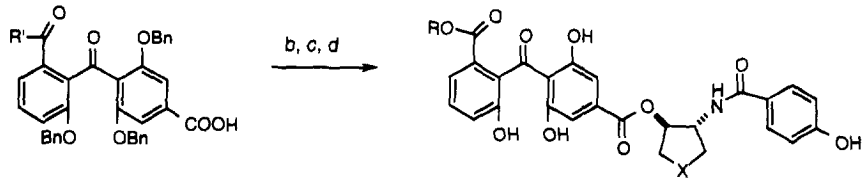


2 X = NH
3 X = CH_2

The esters investigated were chosen for their variety in size and potential prodrug activity. Three basic synthetic approaches were used in the preparation of these esters. For simple esters, Corey's procedure for converting an aldehyde to an ester *via* a cyanohydrin intermediate was used.[5] Treatment of aldehyde **4**[6] with MnO_2, CH_3COOH and KCN in either methanol or isopropanol resulted in the formation of the corresponding methyl (**5**) or isopropyl (**6**) ester of the benzophenone portion (Scheme 1). Coupling to either the cyclopentyl amino alcohol or the pyrrolidine subunit[7] followed by deprotection yielded balanol esters **7**, **8** and **9**.

Esters **15-20** were prepared by alkylating acid intermediates **10**[8] and **3**, where the nitrogen of the pyrrolidine ring was protected either as the benzyloxycarbonyl (CBZ) or *t*-butoxycarbonyl (BOC) carbamate

Scheme 1



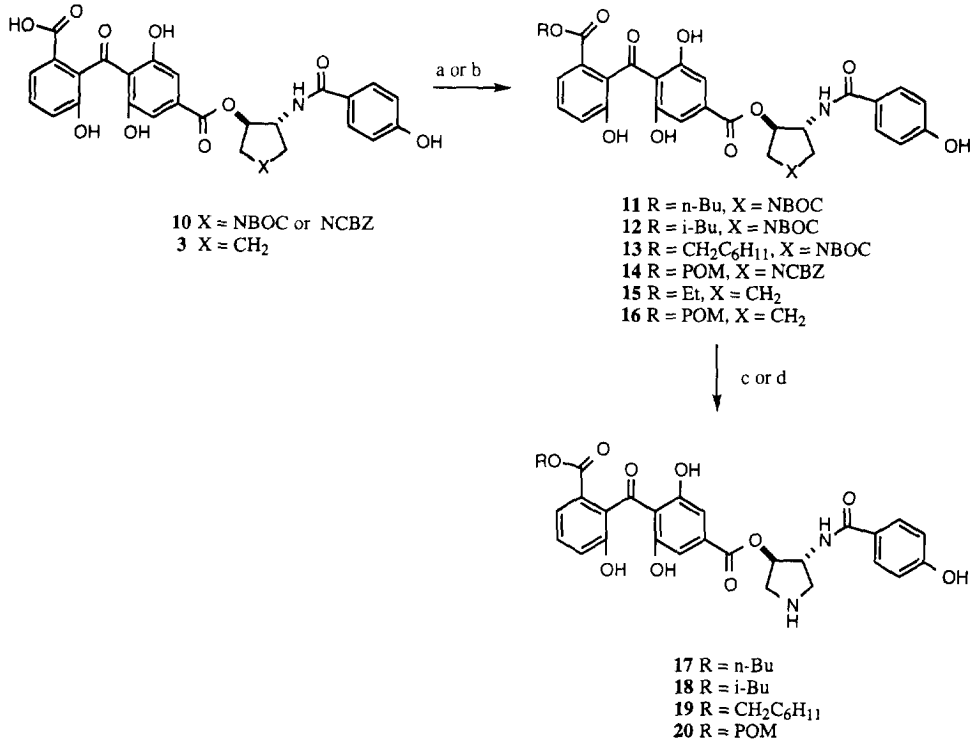
a: **4** R' = H; **5** R' = OMe; **6** R' = OiPr

7 R = Me, X = NH
8 R = Me, X = CH_2
9 R = iPr, X = CH_2

Reagents: a. MnO_2, KCN, CH_3COOH, ROH, r.t. (38-52%). b. $(COCl)_2$, DMF, CH_2Cl_2, 0 °C. c. Et_3N, DMAP, (±)-*anti*-2-(4-benzyloxybenzamido)-1-hydroxycyclopentane or (±)-N-CBZ-*anti*-3-hydroxy-4-(4-benzyloxybenzamido)pyrrolidine (76% from **5** or **6**). d. H_2 (1 atm), TFA, $Pd(OH)_2$ / C, MeOH, MeOAc (70%).

(Scheme 2). Alkylation using sodium bicarbonate in DMF resulted in moderate yields of the desired ester products with little alkylation of any benzophenone hydroxyls. Interestingly, the use of sodium carbonate resulted in a significant amount of O-alkylation. Deprotection of pyrrolidine intermediates **11-14** using standard reaction conditions yielded the pyrrolidine analogs **17-20**.

Scheme 2



10 X = NBOC or NCBZ
3 X = CH_2

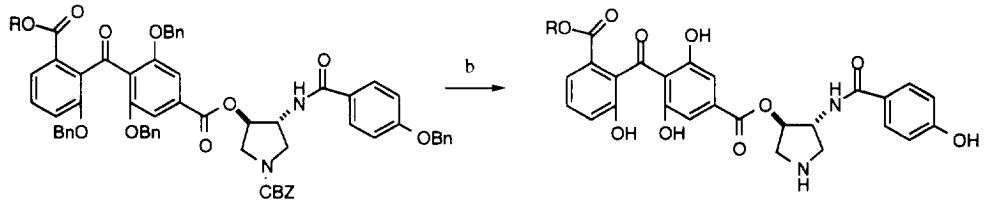
11 R = n-Bu, X = NBOC
12 R = i-Bu, X = NBOC
13 R = $CH_2C_6H_{11}$, X = NBOC
14 R = POM, X = NCBZ
15 R = Et, X = CH_2
16 R = POM, X = CH_2

17 R = n-Bu
18 R = i-Bu
19 R = $CH_2C_6H_{11}$
20 R = POM

Reagents: a. R-I, $NaHCO_3$, DMF (23-44%). b. R-Br, NaI, $NaHCO_3$, DMF (7-50%). c. TFA (58-92%). d. H_2 (1 atm), $Pd(OH)_2$ / C, EtOH, EtOAc (34%).

Due to interference of acidic phenolic hydroxyl groups, the acetoxymethyl ester, **24**, could not be formed *via* alkylation of intermediate **10**. Instead, intermediate **21**[9] (where all phenolic hydroxyls are protected) was alkylated using bromomethyl acetate and sodium carbonate in DMF followed by deprotection to yield the desired product, **24** (Scheme 3). Ethyl ester **23** was derived from alkylation of the benzophenone subunit with ethyl iodide prior to coupling with the pyrrolidine subunit.

Scheme 3



a → **21** R = H; **22** R = CH_2OCOCH_3; **23** R = Et

24 R = CH_2OCOCH_3
25 R = Et

Reagents: a. bromomethylacetate, Na_2CO_3, DMF, acetone (82%). b. H_2 (1 atm), $Pd(OH)_2$ / C, EtOH, EtOAc, TFA (60-75%).

Balanol analogs were screened against eight known isozymes (α, βI, βII, δ, γ, ε, η, ζ)[10] as well as in a number of cellular assays. We selected as our primary cellular assay the neutrophil assay, which measures inhibition of a phorbol-induced superoxide generation in human neutrophils and serves as a model system for studying cellular responses that involve PKC.[11,12] Tables 1 and 2 summarize the data showing a representative selection of the isozyme data. Isozymes not shown showed similar trends in activity.

Entries **7**, **25**, **17**, **18**, and **19** in Table 1 show that increasing the size of the R group resulted in decreased enzyme inhibitory activity. This trend is evident in the cyclopentyl series (**8**, **15**, **9**, Table 2) as well. Stability studies[13] on (±)**8**, **7**, and **25** indicated that these esters were relatively stable to plasma; less than 20% hydrolysis was observed over a 2 hour period. Butyl esters **17** and **18** showed no evidence of degradation after two hours of incubation. Therefore, it is unlikely that these esters were hydrolyzing during the course of the assay and as a result, these alkyl esters are not believed to be acting *via* a prodrug mechanism. While none of the esters in the pyrrolidine series (except **24**) exhibited cellular activity, the esters in the cyclopentyl series (**8**, **15**, **9**) showed good cellular activity. In addition to the masking of the polar acid group with an ester function, the cyclopentyl series has the added advantage that the basic nitrogen in the central ring is absent. With both charged functionalities gone, the adduct may be more easily transported into the cell. Indeed the active methyl ester enantiomer, (-)**8**, showed enzyme inhibitory activity close to that of balanol in addition to cellular activity, which balanol lacks.

Both pivaloyloxymethyl (POM) and acetoxymethyl (AOM) esters (**16**, **20**, **24**) were designed to act as prodrugs, staying intact long enough to allow entry into the cell and hydrolyzing once in the cell to release the active carboxylic acid (**2** or **3**). These types of esters have been shown to be readily hydrolyzed under the influence of esterases present in serum.[14] Surprisingly, both the POM and AOM esters of the pyrrolidine analog

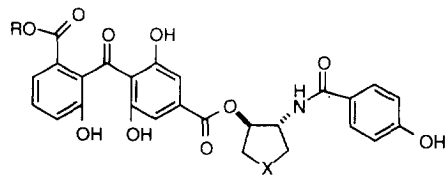


Table 1

compound	X	R	PKC enzyme assays					neutrophil
			α	βII	δ	ε	η	assay
1	$-(CH_2)_2NH-$	H	0.074	0.044	0.032	0.049	0.022	>10
2	NH	H	0.022	0.033	0.005	0.01	0.004	>50
7	NH	CH_3	0.25	0.29	0.018	0.17	0.016	>50
25	NH	Et	2.2	1.0	0.05	1.3	0.02	>10
17	NH	n-Bu	3.1	2.2	0.08	3.7	0.04	>10
18	NH	i-Bu	19	20	0.20	3.2	0.05	>10
19	NH	$CH_2C_6H_{11}$	17	22	0.31	25	0.05	>10
20	NH	POM	0.16	0.02	0.010	0.034	0.004	2.8-10[a]
24	NH	AOM	0.13	0.02	0.024	0.13	0.014	>10[a]

Values given as IC_{50}'s in μM. Representative isozyme data shown. All analogs are racemic. [a] 1 hour preincubation at 4 °C.[15]

(**20**, **24**) showed good enzyme inhibitory activity as compared to simple alkyl esters of comparable size such as **18** and **19**. One possible explanation for this observation may be that the acyloxymethyl esters hydrolyzed rapidly under the conditions of the enzyme assay and, thus, released the active acid into the assay medium. Conversely, if the ester stayed intact, the acyloxymethyl group itself may have been interacting with the binding site in such a way as to cause increased activity. Neutrophil assays on these compounds employed a one hour incubation prior to running the assay to provide a greater opportunity for the test compounds to equilibrate across the cellular membrane.[15] The POM ester (**20**) showed cellular activity while the AOM ester (**24**) did not. The AOM ester has been reported to be more labile than the POM and may have hydrolyzed faster than it could be transported into the cell.[14]

Similarly, the POM ester of the cyclopentyl analog (**16**) exhibited good cellular activity. The increased lipophilicity of **16** due to the lack of the basic nitrogen may be responsible for the increased activity of **16** over **20**. Table 2 indicates two sets of cellular data for compounds **3**, (±)**8**, and **16**. These show neutrophil data with and without a one hour preincubation. As expected, both **3** and (±)**8** showed no difference when the preincubation was employed. However, the cellular activity of the POM ester **16** increaseed 10-fold with the preincubation, suggesting that it did indeed act as a prodrug in this assay.

In conclusion, the series of esters described herein shows that masking the carboxylic acid functionality of balanol analogs **2** and **3** with a small group such as methyl can result in analogs with good enzyme inhibitory activity while larger alkyl groups interfere with PKC interaction. In the presence of the basic nitrogen (pyrrolidine analogs) only the POM ester showed cellular activity, presumably acting *via* a prodrug mechanism. In the cyclopentyl series, the POM ester, as well as small alkyl esters such as methyl and ethyl,

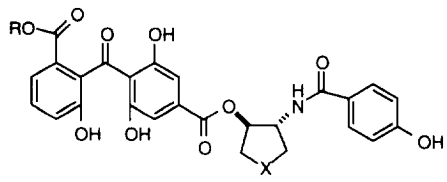


Table 2

compound	X	R	PKC enzyme assays					neutrophil
			α	βII	δ	ε	η	assay
3	CH_2	H	0.04	0.05	0.0009	0.05	0.0006	>10, >10[a]
(±)8	CH_2	Me	0.32	0.33	0.03	0.26	0.03	0.8-2.1, 1.72-3.92[a]
(+)8	CH_2	Me	4.2	1.8	0.25	20	0.4	>10
(-)8	CH_2	Me	0.21	0.04	0.005	0.30	0.004	1.5
15	CH_2	Et	0.92	1.6	0.067	0.66	0.052	2.6
9	CH_2	iPr	4.7	0.44	0.05	13	0.26	10
16	CH_2	POM	na	na	na	na	na	3.68-5.32, 0.33-0.85[a]

Values given as IC_{50}'s in μM. Representative isozyme data shown. All analogs are racemic unless otherwise indicated. [a] 1 hour preincubation at 4 °C.[15]

showed good cellular activity. Thus, this study has shown that PKC inhibitory activity can be maintained and cellular activity increased by masking the polar acid functionality of cyclopentyl and pyrrolidine balanol analogs.

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8. Compound **10** (N-CBZ) resulted from the coupling of the fully protected benzophenone, 4-[4-(2-benzyloxy-6-(benzyloxycarbonyl)benzoyl]-3,5-dibenzyloxybenzoic acid,[6] with (±)-N-CBZ-*anti*-3-hydroxy-4-(4-benzyloxybenzamido)pyrrolidine[7] using the same method as the transformation shown in Scheme 1 for the formation of analog **7**. Compound **10** (N-BOC) resulted from the above conditions except the benzophenone acid was coupled to (±)-N-BOC-*anti*-3-hydroxy-4-(4-benzyloxybenzamido)pyrrolidine which was obtained in the same manner as (±)-N-CBZ-*anti*-3-hydroxy-4-(4-benzyloxybenzamido)pyrrolidine, from 3-pyrroline.[7]

9. Compound **21** resulted from the coupling of **4** to (±)-N-CBZ-*anti*-3-hydroxy-4-(4-benzyloxybenzamido)-pyrrolidine[7] using carbonyldiimidazole in DMF. This was followed by oxidation using sulfamic acid and sodium chlorite in acetonitrile and water. Lindgren, B. O.; Nilsson, T. *Acta Chem. Scand.* **1973**, *27*, 888.

10. Human PKC enzymes were expressed in Sf-9 cells and paritially purified. Addition of protein to the substrate in vesicles consisting of 120 μg/mL phosphatidylserine, diacylglycerol (varying amounts) in 20 mM HEPES buffer (pH = 7.5), 10 mM $MgCl_2$, 200 μg/mL histone (type HL), 925 μM $CaCl_2$, 1.0 mM EGTA and 30 μM gamma ^{32}P-ATP were incubated at 30 °C for 10 minutes, and the reaction was then quenched by addition of 0.5 mL of ice cold Cl_3CCOOH. The precipitate was collected and radioactivity measured. IC_{50}'s were determined using a 4 point curve of 10-fold dilutions.

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12. Phorbol-12-myristate-13-acetate (PMA, 15 ng/mL) was added to lucigenin in reaction HBSS containing a neutrophil suspension (2 x 10^6 cells / mL). Cuvettes were loaded into a luminometer and chemiluminescence at 550 nm was measured for 15 cycles at 37 °C. Determinations made in the presence of test compounds were compared to maximum response values. IC_{50}'s were determined using a 4-point curve of 10-fold dilutions.

13. Stability studies were run in the following manner: Compounds of interest (10 μL of a 5 μmol/mL solution in methanol) were added to human plasma (0.4 mL) at a concentration of 125 μM and incubated at 37 °C. Stability was measured at times of 0, 10, 30, 60 and 120 minutes. After the appropriate time, samples were quenched by treating with methanol (0.8 mL) then centrifuged, after which an aliquot (25 μL) of the plasma supernatant was injected onto an HPLC for quantification of the parent compound and detection of any peaks resulting from hydrolysis products. Methyl ester **7** showed 6% degradation to the acid **2** after 1h incubation and 20% after 2h. Ethyl ester **25** showed 5% degradation to the acid **2** after 2 h incubation. Methyl ester (±)**8** showed 7% degradation to the acid **3** after 1 h and 17% after 2 h. Butyl esters **18** and **19** showed no detectible degradation after 2 h incubation. Degradation products were determined to be the corresponding acids by comparison with authentic acid **2** or **3**. Acids **2** and **3** also showed no degradation in plasma after 2 h incubation.

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15. Compounds of interest were added to cells and incubated at 4 °C for 1 h after which the assay proceeded as described above for the neutrophil assay.

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