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PROTEIN KINASE C INHIBITORY ACTIVITIES OF BALANOL ANALOGS BEARING CARBOXYLIC ACID REPLACEMENTS[1]

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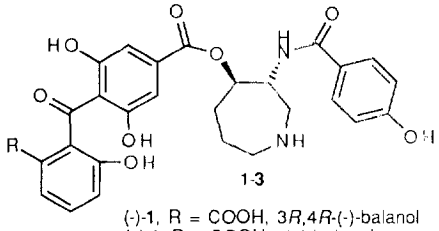
Abstract: A variety of balanol analogs bearing carboxylic acid replacements (amides, sulfonamides and tetrazoles) were synthesized and evaluated for protein kinase C (PKC) inhibitory activity. In general, those compounds which bear an acidic proton (pKa ≤ 7.6) display potent PKC activity, and show selectivity for PKC over other kinases. Prodrugs are excellent tools for increasing cellular activity of some acid replacements.

The complex pathways of cellular signal transduction are of great interest to scientists as potential therapeutic targets. The protein kinase C (PKC) family of enzymes, central to many signal transduction pathways, plays an important role in cellular proliferation and gene expression.[2] As a result, PKC is recognized as an interesting target for the treatment of diseases such as cancer, cardiovascular disorders and asthma.[2c,d]

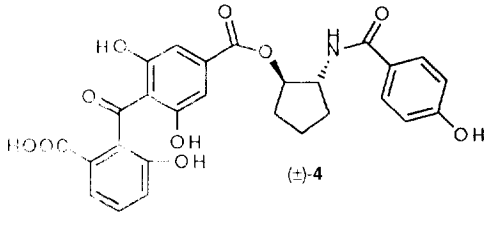
(-)-Balanol, (-)-**1**, isolated recently as a metabolite produced by the fungus *Verticillium balanoides*, was shown to be one of the most potent naturally occurring PKC inhibitors, with IC_{50} values in the low nanomolar range for most isozymes.[3] Its potent activity, combined with its unique and challenging structure, presented an intriguing opportunity for structure activity relationship and synthetic studies.[4]

Early in our balanol analog investigations it became apparent that an important feature of the benzophenone region of the molecule was the carboxylate moiety. If the carboxylic acid was replaced by a simple proton (**2**) or hydroxyl group (**3**), PKC inhibitory activity dramatically decreased.[5] While this acidic functionality appeared to be necessary for enzyme inhibition, its polar nature was thought to limit cell permeability. To this end, studies were undertaken to synthesize a variety of carboxylic acid replacements, namely carboxylic acid bioisosteres and prodrugs, and to evaluate their PKC and cellular activities.



1-3

(-)-**1**, R = COOH, 3*R*,4*R*-(-)-balanol
(±)-**1**, R = COOH, (+)-balanol
(±)-**2**, R = H
(±)-**3**, R = OH



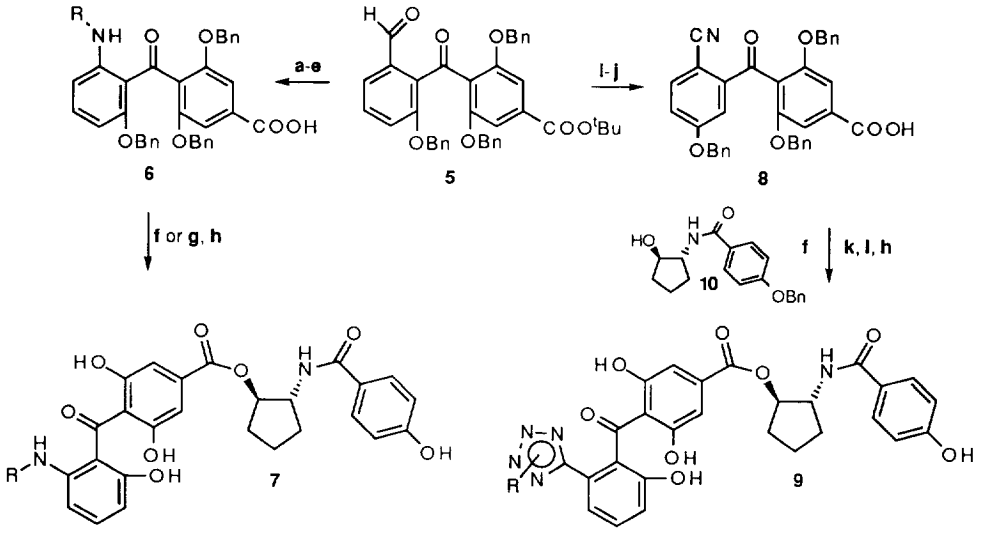
(±)-**4**

It was discovered that a synthetic analog (**4**) utilizing a cyclopentane nucleus showed comparable (1-50 nm) PKC inhibitory activities to that of racemic balanol, ((±)-**1**), which bears an azepine ring.[5] This modification allowed for easier synthetic access to balanol analogs bearing the carboxylic acid replacements mentioned above. In addition, the polarity of the molecule was decreased somewhat, perhaps helping to increase cell permeability.

As an initial investigation, a group of aniline-derived carboxylic acid isosteres was prepared. Aldehyde **5**[6] was transformed, *via* a Curtius rearrangement, to the corresponding aniline which was then acylated or

sulfonylated to give **6**. Coupling to (±)-*trans*-2-(4-benzyloxybenzamido)-1-hydroxycyclopentane, (**10**), followed by deprotection of the benzyl ethers provided balanol analogs **7**.

Scheme



a: $NaClO_2$, H_2NSO_3H; **b**: $(C_6H_5O)_2P(O)N_3$, 95 °C, then $(CH_3)_3SiCH_2CH_2OH$; **c**: CsF, DMF; **d**: acylation with $(RCO)_2O$, RCOCl or RSO_2Cl; **e**: HCOOH; **f**: $(ClCO)_2$, DMF, CH_2Cl_2, then (±)-*trans*-2-(4-benzyloxy benzamido)-1-hydroxycyclopentane (**10**), $^iPr_2NEt$, DMAP, CH_2Cl_2; **g**: **10**, CDI, DBU, DMAP, CH_2Cl_2; **h**: H_2 (1 atm), $Pd(OH)_2$, EtOH or THF; **I**: NH_2OH•HCl, DMF, 60 °C; **j**: CF_3COOH, CH_2Cl_2; **k**: $^nBu_2SnO$, $(CH_3)_3SiN_3$, toluene, 75 °C; **l**: RI, Na_2CO_3, DMF, acetone

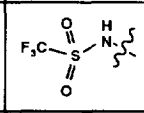
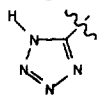
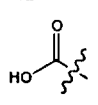
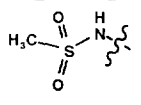
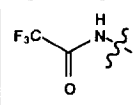
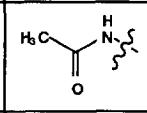
A second family of carboxylic acid isosteres investigated was the tetrazoles. Conversion of aldehyde **5** to the nitrile followed by ester cleavage and coupling to **10** provided a fully protected intermediate. Formation of the tetrazole under known conditions,[7] alkylation if desired, and then benzyl cleavage provided tetrazoles **9**. Satisfactory analytical data (IR, ^{1}H-NMR, FAB-MS and elemental analysis) were obtained for all analogs.

A variety of biological screens were run on the analogs synthesized. Inhibitory enzyme assays consisted of screening against eight human isozymes (α, β–I, β–II, δ, ε, γ, η and ζ) of PKC,[8] as well as other protein kinases (PKA, cyclic-AMP dependent protein kinase and calcium calmodulin dependent protein kinase) to ascertain kinase selectivity. A variety of cellular assays were also conducted including a neutrophil assay, a cellular test which measures the phorbol-12-myristate-13-acetate (PMA) induced release of superoxide in human neutrophils.[9] As this process is thought to be mediated by PKC, it provides one measure of cellular PKC inhibitory activity. Biological data are summarized below; PKC isozymes detailed are representative of trends seen for the families and PKA data are illustrative of other protein kinases screened.

The biological data for those replacements bearing an acidic proton are presented in Table 1, along with the results for carboxylic acid **4** for comparison. In general, PKC inhibitory activity of these new analogs is closely related to the approximate pKa of the acidic proton. Trifluoromethylsulfonamide **7a** and tetrazole **9a**, each quite similar in pKa to acid **4**,[10] retain potent PKC activity and display encouraging selectivity for PKC over PKA. The methylsulfonamide analog **7b**, with a pKa of ~7.6,[11] is 30-40% charged at the physiological pH of

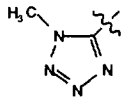
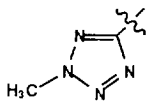
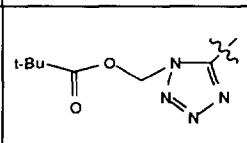
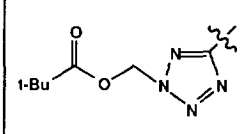
plasma; this analog retains good PKC activity and shows an increased activity in the neutrophil assay. Analogs **7c** and **7d**, both of which have a significantly higher pKa than lead compound **4**, display dramatically decreased PKC activity both in the enzyme and whole cell assays.

Table 1. Biological Data For Replacements Bearing an Acidic Proton (values are expressed as IC_{50}'s (μM))

compound	**7a**	**9a**	**4**	**7b**	**7c**	**7d**
RNH =						
~pKa	4.5	5	5	7.6	9.5	13
PKC-α	0.10	0.34	0.04	0.45	>50	>50
PKC-βII	0.05	0.29	0.05	0.15	46	4.8
PKC-δ	<0.05	0.02	0.0009	0.03	4.4	0.42
PKC-ε	0.31	2.0	0.05	2.0	>50	32
PKA	4.6	1.1	0.3	4.3	>50	29
neutrophil	>10	>10	>10	~10	>10	>10

Biological data for the substituted tetrazoles **9b-e** (i.e. those on which the acidic proton is masked at the time of introduction into the assay) are summarized in Table 2. In general, these substituted systems are less active against PKC than the acidic analogs, however, selectivity for PKC over other kinases such as PKA is

Table 2. Biological Data For Non-Acidic and ProDrug Replacements (values are expressed as IC_{50}'s (μM))

compound	**9b**	**9c**	**9d**	**9e**
R =				
PKC-α	11	3.6	14	37
PKC-βII	3.5	1.3	2.5	9.1
PKC-δ	0.13	0.09	0.30	2.0
PKC-ε	4.7	3.8	0.64	2.9
PKA	>50	24	>50	>50
neutrophil	10.4	>10	0.85	0.15

retained. The pivaloyloxymethyl (POM) tetrazoles (**9d-e**), which were designed as prodrugs of **9a**, potently inhibit PMA-induced superoxide release in human neutrophils with a standard thirty minute incubation time. This display of cellular activity, as compared to the inactive tetrazole **9a**, suggests that the hydrolyzable POM substituent is indeed allowing the molecule better access to the cellular target. The fact that **9d-e**, which are comparable in enzyme enhibitory activity to methyl tetrazoles **9b-c**, are nonetheless more potent in the cellular assay further indicates that **9d-e** are acting through a prodrug mechanism. This prodrug study was based on detailed stability studies of aryl POM-tetrazoles available in the literature for both aqueous buffer solutions and

plasma. The plasma stability in those studies showed half-lives for both the 2-POM and 3-POM tetrazoles to be approximately 1.5 and 3.5 minutes, respectively,[12] thus suggesting that **9d-e** would indeed hydrolyze under the conditions of our assay.

In conclusion, balanol analogs with carboxylate replacements bearing an acidic proton, particularly trifluoromethylsulfonamide **7a** and tetrazole **9a**, display excellent inhibition of PKC and good selectivity for PKC over other kinases. Methylsulfonamide **7b**, partially charged at physiological pH, is not only active against and selective for PKC, but also displays modest activity in the neutrophil assay, a marked contrast to its fully charged (**4**, **7a**, **9a**) or neutral (**7c-d**, **9b-e**) counterparts. Substituted tetrazoles **9b-e** are generally less active against PKC but still maintain selectivity for PKC over PKA. Pivaloyloxymethyl tetrazoles **9d-e** behave well as prodrugs of acid replacement **9a**, thus providing two of the most potent balanol analogs examined in cellular assays to date.

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References and Notes:

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8. Human PKC enzymes were expressed in Sf-9 cells and partially 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, was followed by incubation at 30 °C for 10 minutes and quench of the reaction by addition of 0.5 mL of ice cold Cl_3CCOOH. The precipitate was collected and the radioactivity measured. IC_{50}'s were determined using a 4 point curve of 10-fold dilutions. For further details, see: Kulanthaivel, P.; Janzen, W. P.; Ballas, L.M.; Jiang, J.; Hu, C.; Darges, J. W.; Seldin, J.; Cofield, D.; Adams, L. *Planta Medica* **1995**, *61*, 41.
9. Phorbol-12-myristate-13-acetate (PMA, 3 ng/mL final concentration) was added to lucigenin (50 μM final concentration) in reaction HBSS containing a human neutrophil suspension (4 x 10^6 cells/mL). Cuvettes were loaded into a lumninometer and chemiluminescence at 550 nm was measured for 15 cycles at 37 °C. The PMA concentration which gave near maximal superoxide release was determined; the above sequence was then repeated in the presence of the test compound. IC_{50}'s were determined using a 4 point curve of 10-fold dilutions.
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