modifications of the side chain at position 21 have little effect on binding to FKBP (e.g., FK-506 vs ascomycin),[17] suggesting that this portion of the molecule may not be involved in binding to the protein. This is consistent with the lack of NOEs between the ethyl side chain of ascomycin and FKBP. It is interesting to note that those portions of ascomycin that were found to be in close proximity to FKBP from the NOE data are the same parts of the molecule in common with rapamycin, a potent immunosuppressant which also binds tightly to FKBP. Indeed, as noted previously,[14] the piperidine ring of rapamycin and FK-506 is involved in binding to FKBP and is likely to be oriented in a similar manner in the two ligand/receptor complexes.

Conclusions. From an analysis of NOEs observed in heteronuclear 3D NOE spectra of the [U-^{13}C]ascomycin/FKBP complex, the conformation of ascomycin when bound to its putative target protein, FKBP, was determined. The conformation of ascomycin was found to be very different from the X-ray structure of FK-506 determined in the absence of FKBP, but more closely resembles that of uncomplexed rapamycin. In addition, from the observation of ascomycin/FKBP NOEs in the 3D spectra, those portions of ascomycin that are in close proximity to FKBP were identified, and they include parts of the piperidine, pyranose, and cyclohexyl rings. By using the structural information obtained from this study it may be possible to design ascomycin analogues that are structurally dissimilar from ascomycin but which nonetheless maintain the necessary functionality in the proper orientation for binding to FKBP. It is hoped that analogues could be designed with improved physicochemical properties and potentially lower toxicity, thus making them superior immunosuppressive agents.

During the preparation of this manuscript, a report on the X-ray crystal structure of the FK-506/FKBP complex appeared.[23] The conformation of ascomycin when bound to FKBP as determined by NMR is very similar to the conformation of FK-506 when bound to FKBP as determined by X-ray crystallography. In both structures of the bound ligands, the 9,10 amide bonds are trans and the relative orientation of the pyranose and cyclohexyl rings are the same. In the X-ray structure roughly 50% of the ligand surface is buried at the protein–ligand interface with the region around the allyl and cyclohexyl groups being exposed to solvent. We observed no NOEs from the ethyl group of ascomycin (analogous to the allyl group in FK-506) to the protein, which is consistent with this portion of the molecule being exposed to solvent. However, we do observe several NOEs from the cyclohexyl ring (Figure 5) to the protein, which clearly indicates that a portion of this ring is in close proximity to the protein in solution.

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Supplementary Material Available: A table of ^{1}H and ^{13}C chemical shifts of [U-^{13}C]ascomycin bound to FKBP (1 page). Ordering information is given on any current masthead page. The coordinates of ascomycin when bound to FKBP will be deposited in the Brookhaven Protein Data Bank.

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Substituted 2-(Aminomethyl)piperidines: A Novel Class of Selective Protein Kinase C Inhibitors

Since its discovery in 1977, the ubiquitous enzyme protein kinase C (PKC)[1] has received extensive pharmacological investigation and has emerged as a pivotal mediator in cellular regulation, signal transduction, and neoplastic promotion. Physiological activity of the enzyme is regulated by the allosteric modulators Ca^{2+}, diacylglycerol (DAG), and phosphatidylserine (PS).[1,2] These modulators interact with the enzyme's regulatory domain while the catalytic domain possesses the site of ATP and substrate binding.[3] Phorbol esters have been found to substitute for diacylglycerol as a potent enzyme stimulator,[4] and in animals, phorbol ester activation of PKC causes intense inflammation. Unlike diacylglycerol, phorbol esters are not rapidly metabolized and thus can effect prolonged enzyme stimulation often leading to neoplastic events.[5]

Given the biological responses induced by activators of protein kinase C, the development of inhibitors of this enzyme may lead to therapeutic agents useful in the treatment of chronic inflammatory and proliferative diseases. Several natural and synthetic agents have been identified as PKC antagonists. These include (1) nonselective phospholipid competing agents such as triphenylethylenes,[6] chlorpromazine,[7] and trifluoperazine,[8] (2)

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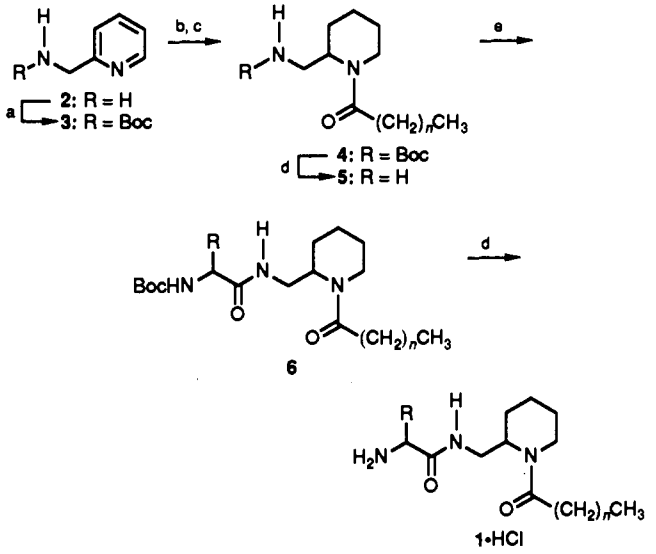
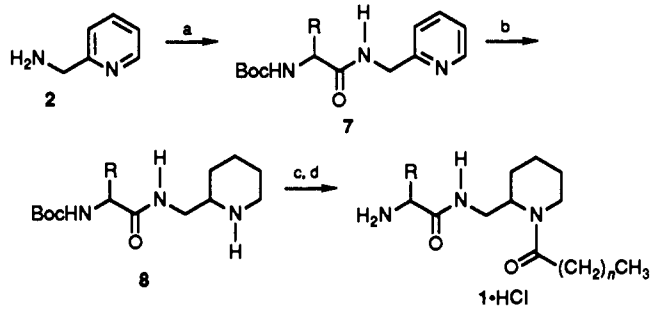
Scheme I[a]



[a] (a) Boc_2O, aq NaOH, THF, 0 °C to room temperature; (b) H_2, PtO_2, HOAc, EtOH, 60 psi; (c) $CH_3(CH_2)_nCOCl$, pyr, DMAP, CH_2Cl_2, 0 °C to room temperature or $CH_3(CH_2)_nCO_2H$, EDC, DMAP, CH_2Cl_2, room temperature; (d) anhydrous HCl, dioxane; (e) L- or D-BocHNCH(R)CO_2H, EDC, HOBt·H_2O, NMM, CH_2Cl_2, 0 °C to room temperature.

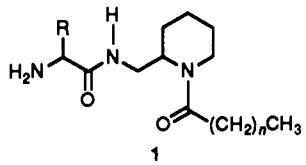
peptide analogues[9] which lack utility due to in vivo instability, (3) agents interacting with the ATP binding site of the enzymes catalytic domain including staurosporine[10] and isoquinolinesulfonamides[11] which also interact with the highly homologous domains of cGMP- and cAMP-dependent kinase, thus limiting their usefulness as specific PKC inhibitors, and (4) inhibitors interacting with the enzymes regulatory domain such as lipoidal amines,[12] aminoacridines,[13] and calphostin C,[14] the most potent, selective PKC inhibitor known to date.

Scheme II[a]



[a] (a) L- or D-BocHNCH(R)CO_2H, EDC, HOBt·H_2O, NMM, CH_2Cl_2, 0 °C to room temperature; (b) H_2, PtO_2, HOAc, EtOH, 60 psi; (c) $CH_3(CH_2)_nCOCl$, pyr, DMAP, CH_2Cl_2, 0 °C to room temperature or $CH_3(CH_2)_nCO_2H$, EDC, DMAP, CH_2Cl_2, room temperature; (d) anhydrous HCl, dioxane.

During our course of investigations relating to the development of novel antiinflammatory agents, piperidinylamides of the general structure 1 were discovered to possess selective PKC inhibitory activity. In this communication, we report the synthesis and preliminary pharmacological evaluation of a series of analogues based on structure 1.



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Scheme I (method A)[15] depicts the synthesis of our general series. Catalytic hydrogenation of *N*-Boc[16] protected 2-(aminomethyl)pyridine (3) over PtO_2 followed by acid chloride treatment or carbodiimide coupling[17] with a carboxylic acid efficiently produced racemic amides 4. Acidic removal of the *N*-Boc protecting group yielded amino amides 5. This sequence provides common intermediates for derivativization and is amenable to large-scale preparation. Coupling of enantiomerically pure *N*-Boc-protected amino acids with racemic intermediate 5 employing EDC/HOBt[18] and subsequent amine deprotection with HCl in dioxane under anhydrous conditions provided the diastereomeric targets 1 as hydrochloride salts.

Alternatively, a more direct route as outlined in Scheme II (method B)[15] can be employed. Conventional carbodiimide coupling[18] of enantiomerically pure *N*-Boc-protected amino acids with 2-(aminomethyl)pyridine (2) gave the desired chiral amides 7, which readily undergo catalytic hydrogenation to provide the piperidinylamides 8 as a mixture of diastereomers. Amidation of the piperidine nitrogen as previously described and removal of the *N*-Boc protecting group with anhydrous HCl in dioxane yielded the hydrochloride salts of the desired diastereomeric targets 1.

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(15) All new compounds reported herein exhibit ^1H NMR, IR, and elemental analysis (within 0.4% of the calculated value) in agreement with the assigned structures.

(16) Abbreviations used are as follows: Boc, *tert*-butoxycarbonyl; DMAP, 4-(*N*,*N*-dimethylamino)pyridine; EDC, 1-[3-(dimethylamino)propyl]-3-carbodiimide hydrochloride; HOBt, 1-hydroxybenzotriazole; NMM, 4-methylmorpholine.

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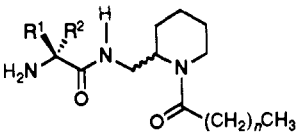
Table I. Substituted 2-(Aminomethyl)piperidines and Their Inhibition of PKC versus PKA



compound	R^1	R_2	n	method[a]	PKC inhibition: IC_{50}, μM[b]	PKA inhibition: % at 100 μM
staurosporine					0.0027	IC_{50} = 27 nM
sphingosine					57 ± 5	IC_{50} = 98 μM
5			12	A	36 ± 2	46 ± 24
9	CH_3	H	11	A	52 ± 6	0
10	$CH_2CH(CH_3)_2$	H	11	A	154[c]	0
11	$CH_2CH_2SCH_3$	H	11	A	159[c]	0
12	CH_2Ph	H	11	A	agonist	
13	H	H	11	A	51 ± 4	0
14	CH_2OH	H	11	A	45 ± 5	0
15	$CH_2CH_2C(O)NH_2$	H	11	A	72 ± 13	0
16	$CH_2CH_2CO_2Bn$	H	11	A	92 ± 12	0
17	$CH_2CH_2CO_2H$	H	11	A	inactive	0
18	CH_2Im	H	11	A	60 ± 6	0
19	$(CH_2)_3NH_2$	H	11	A	23 ± 7	0
20	$(CH_2)_4NH_2$	H	11	A	20 ± 3	0
21	$(CH_2)_3NHC(NH)NH_2$	H	11	A	72 ± 9	0
22	H	$(CH_2)_4NH_2$	11	A	34 ± 12	0
23	$(CH_2)_4NH_2$	H	5	B	inactive	0
24	$(CH_2)_4NH_2$	H	7	B	inactive	0
25	$(CH_2)_4NH_2$	H	9	B	299[c]	0
26	$(CH_2)_4NH_2$	H	10	B	102[c]	0
27	$(CH_2)_4NH_2$	H	12	B	48 ± 19	0
28	$(CH_2)_4NH_2$	H	13	B	38 ± 5	0
29	$(CH_2)_4NH_2$	H	14	B	45 ± 1	0
30	$(CH_2)_4NH_2$	H	15	B	46 ± 1	0
31	$(CH_2)_4NH_2$	H	16	B	36 ± 6	38 ± 3

[a] All new compounds exhibit ^1H and IR data in agreement with assigned structures. Elemental analysis for final compounds were within ±0.4% of theoretical values for C, H, N. [b] Concentration–inhibition curves were determined at inhibitor concentrations up to 100 μM. [c] Represents an extrapolated IC_{50} value.

The ability of synthesized compounds **5** and **9–31** to suppress PKC activity was tested in vitro. The inhibition of ^{32}P incorporation into histones by partially purified rat brain PKC in the presence of phorbol myristate acetate (PMA), Ca^{2+}, and phosphatidylserine was assayed.[19] In addition, specificity was assessed by measuring the inhibition of cAMP-dependent kinase (PKA).[20] These results are summarized in Table I.

Several structure–activity relationship observations regarding these analogues can be derived from evaluation of the biological data presented in Table I. First, activity appears to be modulated by the basicity of the amino acid residue as evidenced by comparison of compounds **18–21**. The basicity requirement is optimized by inclusion of the amino acid ornithine or lysine to produce the most potent analogues **19** and **20**, respectively. Incorporation of the weakly basic histidine or the strongly basic arginine residue results in diminished potency. The presence of an acidic functionality as in derivative **17** abolishes biological response. Esterification, however, produces the inhibitory derivative **16**. Omission of the amino acid fragment produces a nonselective antagonist as shown by compound **5**. Second, a long-chain acyl group is essential for activity. Compounds **20** and **23–31** display a direct relationship between PKC inhibitory potency and chain length. Studies with these analogues reveal that alkyl chains shorter than 13 carbons greatly reduce potency and alkyl chains longer than 18 carbons compromise kinase selectivity. Third, the inclusion of bulky nonpolar substituents in the α-chain of the amino acid moiety results in significantly reduced potency as exemplified by analogues **9–12**. In fact, the bulky phenylalanine derivative **12** exhibits agonist activity. Those analogues containing a polar amino acid residue tend to be more effective than their nonpolar counterparts as indicated by compounds **13–16**. Finally, comparison of **20** and **22** suggests the effect of the stereogenic center of the amino acid fragment upon potency is modest.

To investigate the mechanism of action of these analogues, the inhibitory properties of **20** were studied in more detail. Under conditions which elicit maximal protein kinase activity with purified enzyme, **20** selectively inhibited PKC (IC_{50} = 19 ± 2 μM)[21] with no observable inhibition of PKA or calcium/calmodulin-dependent myosin light chain kinase at concentrations up to 100 μM.

Phorbol ester activation of PKC was studied at fixed concentrations of phosphatidylserine (100 $\mu g/mL$) and Ca^{2+} (400 μM) with varying concentrations of **20**. Lineweaver–Burk and Dixon analyses of the data (Figure 1)

(19) Inhibition of histone phosphorylation was measured by the method found in: Huang, K. P.; Nakabayashi, H.; Huang, F. L. Isozymic forms of rat brain Ca^{+2}-activated and phospholipid-dependent protein kinase. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 8535–8539.

(20) Protein kinase A activity was determined in the presence of 20 mM Tris buffer, 10 μM histone, 4 ng/mL of the catalytic subunit of protein kinase A, 100 μM dibutyl-cAMP, and 100 μM [^{32}P]ATP as described in Davis, P. D.; Hill, C. H.; Keech, E.; Lawton, G.; Nixon, J. S.; Sedgwick, A. D.; Wadsworth, J.; Westamcott, D.; Wilkinson, S. E. Potent Selective Inhibitors of Protein Kinase C. *FEBS Lett.* **1989**, *259*, 61–63.

(21) Reported value represents mean ± SEM of four individual determinations.

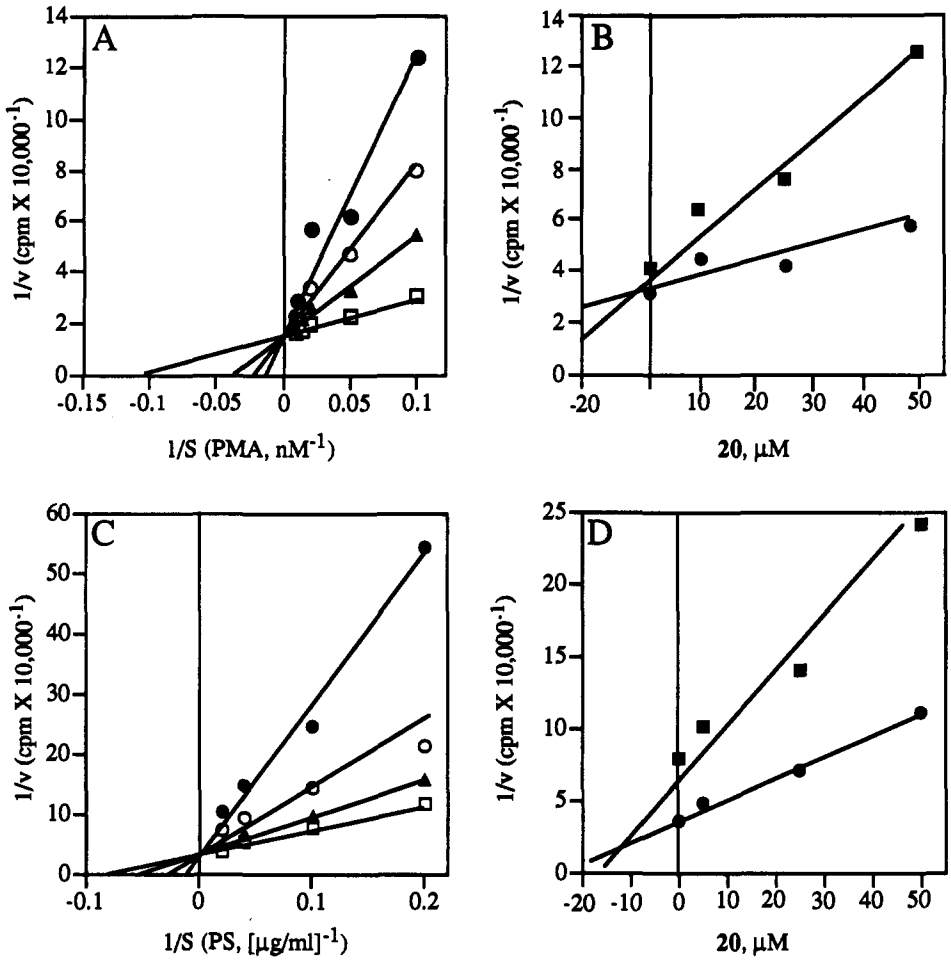


Figure 1. (A) Lineweaver–Burk plot of the effect of PMA on the inhibition of PKC-α by compound **20**. PKC activity was determined in the presence of 400 μM "free calcium", 100 μg/mL PS, 10 μM ATP, and the indicated concentrations of PMA. Compound **20** was present at 0 μM (■), 10 μM (▲), 25 μM (○), and 50 μM (●). (B) Dixon analysis (1/velocity vs concentration of **20**) of the effect of PMA on the inhibition of PKC-α by compound **20**. K_i may be determined from the point of intersection of the two lines.[22] PMA concentrations used were 10 nM (■) and 50 nM (●). (C) Lineweaver–Burk plot of the effect of PS on the inhibition of PKC-α by **20**. PKC activity was determined in the presence of 400 μM "free calcium", 1 μM ATP, and the indicated concentrations of PS. Compound **20** was present at 0 μM (■), 10 μM (▲), 25 μM (○), and 50 μM (●). (D) Dixon analysis (1/velocity vs concentration of **20**) of the effect of PS on the inhibition of PKC-α by compound **20**. PS concentrations were 5 μg/mL (■) and 25 μg/mL (●).

indicate this analogue is a competitive inhibitor with respect to phorbol ester activation (K_i = 5 ± 3 μM).[21] Additionally, phosphatidylserine activation of PKC was competitively inhibited by **20** (K_i = 12 ± 4 μM, Figure 1).[21] Compound **20** also inhibited [^{3}H]phorbol dibutyrate binding (IC_{50} = 23 ± 4 μM) to the enzyme preparation, further suggesting that inhibition of PKC activity by **20** may be the result of interactions with the enzyme's regulatory domain. Recent studies employing recombinant mutant PKC enzymes support evidence that compound **20** interacts with the regulatory domain of the enzyme.[23] Use of the mutant enzymes localized the effect of compound **20** to the N-terminal region of the C-1 domain known to possess the amino acid sequence that includes the pseudosubstrate region and part of the first of the cysteine-rich repeat sequences.

Synthesis of a novel series of selective PKC inhibitors should provide useful tools for elucidating the physiological role of PKC in various cellular processes. The development of these and related agents for therapeutic evaluation in cellular and living systems is currently in progress.

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A Selective, Reversible, Competitive Inhibitor of Monoamine Oxidase A Containing No Nitrogen, with Negligible Potentiation of Tyramine-Induced Blood Pressure Rise

Monoamine oxidase (MAO) (EC 1.4.3.4, amine oxidase, flavin containing) consists of two different forms distinguishable by their substrate specificity[1] and their amino acid sequences.[2] Ratios of MAO A to MAO B of ca. 1 and

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