[^{3}H]metaraminol, 124201-39-4; 6-[^{18}F]fluorometaraminol, 112113-61-8; 6-[^{3}H]metaraminol, 124201-40-7; metaraminol bitartrate, 33402-03-8; 6-[^{125}I]iodometaraminol, 124201-41-8; 4-[^{125}I]iodometaraminol, 124201-42-9.

Supplementary Material Available: Tissue distribution data in rats for the 4- and 6-[^{125}I]iodometaraminols and the [4-^{3}H]- and [6-^{3}H]metaraminols are available (2 pages). Ordering information is given on any current masthead page.

Small Peptide Inhibitors of Smooth Muscle Myosin Light Chain Kinase[1]

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The pentapeptide Ser-Asn-Val-Phe-Ala-OBzl has been identified as the smallest inhibitory peptide of myosin light chain kinase (MLCK) derived from the primary sequence of the light chain phosphorylation site. The specific contributions of individual amino acid side chains and backbone elements of this pentapeptide toward the stabilization of the enzyme–inhibitor (E·I) complex have been evaluated. The potency of these peptides as inhibitors of MLCK has been enhanced by the incorporation of synthetic nonnatural amino acids into the sequence. Finally, it has been demonstrated that these peptide sequences could be converted into pseudopeptides with synthetic nonpeptide subunits designed to mimic peptide bonds, and that certain pseudopeptides retained the high-affinity inhibition of the parent pentapeptides.

Myosin light chain kinase (MLCK) is a Ca^{2+}-calmodulin dependent enzyme, which catalyzes the transfer of the γ-phosphate group of ATP to a Ser[19] residue in the 20 kDa phosphorylatable light chains of myosin (MLC). In smooth muscle, phosphorylation of Ser[19] is a prerequisite for stimulation of myosin ATPase activity and cross-bridge cycling leading to muscle contraction.[3] Thus, specific inhibitors of smooth muscle MLCK are expected to be smooth muscle relaxants, and may be useful as novel therapeutic agents in the treatment of disease states such as hypertension and bronchoconstriction.

Kemp and co-workers have shown that small polybasic peptide fragments from the amino terminus of MLC are effective substrates for the phosphorylation reaction.[4,5] Further, they demonstrated that a critical spatial requirement exists between the phosphorylatable serine and an assembly of basic residues near the N terminus. The smallest peptide that retained substrate characteristics comparable to those of native light chains was the tridecapeptide 1. Shorter peptides such as **2**, which lack the

	11 12 13 14 15 16 17 18 19 20 21 22 23
MLC_{11-23}	-Lys-Lys-Arg-Pro-Gln-Arg-Ala-Thr-Ser*-Asn-Val-Phe-Ala-
1	Lys-Lys-Arg-Pro-Gln-Arg-Ala-Thr-Ser*-Asn-Val-Phe-Ser-NH_2
2	Arg-Ala-Thr-Ser*-Asn-Val-Phe-Ser-NH_2
3	Lys-Lys-Arg-Ala-Ala-Arg-Ala-Thr-Ser*-NH_2
4	Lys-Lys-Arg-Ala-Ala-Arg-NH_2
5	Lys-Lys-Arg-Ala-Ala-Arg-Ala-Thr-Ser*-Asn-Val-Phe-NH_2
6	Lys-Lys-Arg-Ala-Ala-Arg-Ala-Thr-Ser*-Asn-Ala-Ala-NH_2

*phosphorylatable serine

critical cluster of basic residues, manifest a modestly smaller V_{max} but markedly increased $(K_m)_{app}$ values. Interestingly, smaller peptides such as **3** which contain the cationic cluster but lacked residues beyond the phosphorylatable serine were also poor substrates.[6] This effect resulted almost exclusively from a lower V_{max}.

Small peptides such as **3** have $(K_m)_{app}$ that are essentially identical with those of native myosin light chains (MLC), essentially making these inhibitors of the enzyme rather than substrates. These peptides were good inhibitors of MLCK, in the low micromolar range. Recently, hexapeptide **4** was determined to be the minimal inhibitory peptide containing these basic residues.[7]

We[8] and others[9] have demonstrated that polybasic peptide fragments derived from the primary sequence of chicken gizzard MLCK also were very potent inhibitors. Recent evidence suggests that this region of the enzyme is involved in pseudosubstrate autoregulation. These peptides are derived from a region of the kinase probably involved in calmodulin binding and the mechanism by which they inhibit the enzyme appears to be complex. Inhibition occurs not only by binding calmodulin but also by competition of these peptides with MLC for the active site of the enzyme.

MLCK is a Ca^{2+}-calmodulin-dependent kinase, thus it is expected that calmodulin antagonists will be inhibitors. Compounds such as W-7 have been shown to inhibit the enzyme.[10,11] Finally, myosin light chain kinase can be inhibited by an active site mechanism with small nonpeptides which compete with the ATP cosubstrate at the catalytic site. Within this class of inhibitors is 5′-chloro-5′-deoxyadenosine[12] as well as the arylsulfonamide kinase

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inhibitors discovered by Hidaka and co-workers.[13]

In our program we decided to explore structure–activity relationships within the relatively hydrophobic region, which is to the carboxy terminus of the phosphorylatable Ser. Pearson et al. have shown that while both **5** and **6** have K_m values comparable to those of myosin light chains; they exhibit V_{max} values that are 20 and 1100 times lower, respectively.[14] We felt that analogues of these peptides might be found which would bind tighter without being substrates.

Using the primary sequence of MLC proximal to the phosphorylatable Ser^{19} as a template, we constructed an array of compounds to probe structure–activity relationships. We have found that the peptide Ser-Asn-Val-Phe-Ala-OBzl, a benzyl ester derivative of residues 19–23 of MLC, was a good inhibitor of the kinase, having an IC_{50} of 28 μM against the native form of MLCK and an IC_{50} of 21 μM against a calmodulin-independent MLCK, obtained by limited proteolysis of the native enzyme. These results were particularly interesting in light of previous explorations by Kemp and co-workers which demonstrated the important contributions to binding of the basic residues Lys^{11}, Lys^{12}, Arg^{13}, and Arg^{16} in peptide substrates of MLCK.[4,5] Our studies show that a substantial hydrophobic contribution to binding, provided by the benzyl ester moiety, compensates very effectively for the lack of interactions between basic residues and MLCK.

As a consequence of this initial discovery, we have explored the chemistry and biochemistry of Ser-Asn-Val-Phe-Ala-OBzl and related peptides. In the studies described here we have (1) identified the pentapeptide Ser-Asn-Val-Phe-Ala-OBzl as the smallest inhibitory peptide derived from the primary sequence of the light chain phosphorylation site, (2) evaluated the specific contributions of individual amino acid side chains and backbone elements of this pentapeptide toward the stabilization of the enzyme–inhibitor (E·I) complex, (3) enhanced the potency of these peptides by the incorporation of synthetic nonnatural amino acids into the sequence, and (4) demonstrated that these peptide sequences could be converted into pseudopeptides with synthetic nonpeptide subunits designed to mimic peptide bonds, and that certain pseudopeptides retained the high affinity inhibition of the parent pentapeptides.

Results and Discussion

The initial studies evaluated enzyme inhibition of the native form of myosin light chain kinase. In later studies inhibition was measured against a calmodulin-independent form of MLCK obtained by limited proteolysis with trypsin. We have determined that these two enzymes are functionally equivalent and differ primarily in their calmodulin regulation. In order to assure, however, that they responded equivalently to our inhibitors, selected peptides were evaluated against both species of myolin light chain kinase and these results are shown in the tables. Inhibition appears to be equivalent in the cases examined.

Table I summarizes our studies to determine the composition of a minimum-size inhibitory peptide. Starting with Phe^{22}-Ala^{23} we added amino acids which correspond to the light chain sequence on the amino terminus and evaluated the inhibitory properties after each amino acid extension (**7–13**). Peptides were examined as their benzyl esters primarily because of their flexibility in synthesis. However, an unanticipated result was the discovery of the contribution of this benzyl group to a hydrophobic binding interaction which became more completely understood after further investigation (vide infra).

Upon adding residues, starting with Phe^{22}-Ala^{23} benzyl ester, no significant inhibition was observed until the incorporation of the Ser, which corresponds to Ser^{19} in the light chain sequence. Further extension of the chain, up to and including Arg^{16}, did not significantly enhance the inhibitory properties. These results establish **10** as the minimum inhibitory sequence of MLCK with an IC_{50} of 28 μM. This peptide had a similar IC_{50} (21 μM) against the calmodulin-independent form of MLCK, indicating that inhibition is not due to competition with or binding to calmodulin and suggesting inhibition by interaction at the active site of MLCK. Deletion of carboxyl terminal residues from the pentapeptide (**14**, **15**), while retaining the benzyl ester, resulted in substantially diminished inhibitory properties.

We have prepared synthetic peptides modeled on the template of **10** in which individual amino acids were specifically replaced (at Ser, **16–24**; at Asn, **25–31**; at Val, **32–36**; at Phe, **37–45**; and at AlaOBzl, **46–54**) with other residues. The importance of individual amino acid contributions to the structure–activity relationship for enzyme inhibition was evaluated with these compounds.

The data suggest that Ser is important for inhibitory activity. Nonetheless we have shown (vide infra) that Ser is not phosphorylated in several of our best inhibitors. Furthermore, substitution of Thr (**16**) and Cys (**17**) for Ser in the peptides resulted in inhibitors with equal or better inhibition against the native enzyme. One attractive hypothesis was that the Ser or surrogate was hydrogen bonding to a phosphate anion of the γ-phosphate of ATP in the active site. If this were the case, then the introduction of an electrostatic interaction in the form of a salt bridge would serve to enhance the effect and result in an inhibitor with a lower inhibition constant. Thus the unnatural amino acid L-diaminopropanoic acid was substituted for Ser (**18**). The very poor inhibition observed in this compound suggests that the role of the Ser hydroxyl is not in a direct interaction with a phosphate of ATP.

Amino acids such as Met, Pro, and Gly do not present polar functional groups to the active site and are correspondingly less active than the more effective Ser analogues (**19–21**). However, it is interesting to note that the introduction of a hydroxyl group into the Pro, giving 4-hydroxyproline (Hyp), results in a peptide (**22**) with a 4-fold enhancement in binding relative to Pro. This reenforces the observation that the Ser hydroxyl functional group contributes to the inhibition by the pentapeptide.

Isoserine (Ise) and α-methylserine (α-MeSer) were specifically introduced to explore geometric and conformational effects around the Ser hydroxyl group. Ise and α-MeSer impose, respectively, significant structural and conformational perturbations at the serine subsite. Each of these substitutions result in a peptide with dramatically diminished inhibitory properties (**23**, **24**).

In Table I we also summarize data obtained with analogues containing a modified asparagine residue (**25–31**). Initially it was thought that the Asn side chain was essential for inhibitory potency since substitutions of His, Asp, Ser, and Gly for Asn resulted in peptides which were very poor inhibitors. Even the subtle replacement by Gln which affects the position of the side chain carboxamide relative to the backbone resulted in a dramatic loss in

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Table I. Inhibition of MLCK by Ser-Asn-Val-Phe-Ala-OBzl (**10**) and Its Peptide Analogues[a]

	peptide structure	IC_{50}, μM, or % inhibn (concn) native	independent
7	Phe-Ala-OBzl	0% (1000)	
8	Val-Phe-Ala-OBzl	1000	
9	Asn-Val-Phe-Ala-OBzl	800	
10	Ser-Asn-Val-Phe-Ala-OBzl	28	21
11	Thr-Ser-Asn-Val-Phe-Ala-OBzl	15	
12	Ala-Thr-Ser-Asn-Val-Phe-Ala-OBzl	31	
13	Arg-Ala-Thr-Ser-Asn-Val-Phe-Ala-OBzl	38	
14	Ser-Asn-Val-Phe-OBzl	160	
15	Ser-Asn-Val-OBzl	0% (1000)	
16	Thr-Asn-Val-Phe-Ala-OBzl	18	
17	Cys-Asn-Val-Phe-Ala-OBzl	8	
18	Dpr-Asn-Val-Phe-Ala-OBzl	400	
19	Met-Asn-Val-Phe-Ala-OBzl	76	
20	Pro-Asn-Val-Phe-Ala-OBzl	200	
21	Gly-Asn-Val-Phe-Ala-OBzl	37% (100)	
22	Hyp-Asn-Val-Phe-Ala-OBzl	56	
23	Ise-Asn-Val-Phe-Ala-OBzl	14% (100)	
24	D,L-α-MeSer-Asn-Val-Phe-Ala-OBzl	350	
25	Ser-His-Val-Phe-Ala-OBzl	0% (100)	
26	Ser-Asp-Val-Phe-Ala-OBzl	0% (100)	
27	Ser-Ser-Val-Phe-Ala-OBzl	15% (100)	
28	Ser-Gln-Val-Phe-Ala-OBzl	32% (100)	
29	Ser-Gly-Val-Phe-Ala-OBzl	450	
30	Ser-Val-Val-Phe-Ala-OBzl		24
31	Ser-Ala-Val-Phe-Ala-OBzl		18
32	Ser-Asn-Met-Phe-Ala-OBzl	30	
33	Ser-Asn-Leu-Phe-Ala-OBzl	3–30[b]	
34	Ser-Asn-Phe-Phe-Ala-OBzl	50	
35	Ser-Asn-Gly-Phe-Ala-OBzl		17% (300)
36	Ser-Asn-Thr-Phe-Ala-OBzl		190
37	Ser-Asn-Val-Trp-Ala-OBzl	6	
38	Ser-Asn-Val-Tyr-Ala-OBzl	45	
39	Ser-Asn-Val-Cha-Ala-OBzl	12	
40	Ser-Asn-Val-1Np-Ala-OBzl	2	2
41	Ser-Asn-Val-2Np-Ala-OBzl	12	
42	Ser-Asn-Val-Bpa-Ala-OBzl	1	5
43	Ser-Asn-Val-Dpa-Ala-OBal	6	
44	Ser-Asn-Val-Mit-Ala-OBzl	1	
45	Ser-Asn-Val-Dit-Ala-OBzl	7	
46	Ser-Asn-Val-Phe-Ser-OBzl	43	
47	Ser-Asn-Val-Phe-Ala-O$(CH_2)_2$Ph	15	50
48	Ser-Asn-Val-Phe-Ala-O$(CH_2)_4$Ph	19	
49	Ser-Asn-Val-Phe-Ala-NHBzl	15	16
50	Ser-Asn-Val-Phe-Ala-$NHCH_2$Pyr[c]	0% (1000)	5% (300)
51	Ser-Asn-Val-Phe-Ala-OH		0% (300)
52	Ser-Asn-Val-Phe-Ala-NH_2		0% (300)
53	Ser-Asn-Val-Phe-Ala-O$(CH_2)_3CH_3$		185
54	Ser-Asn-Val-Phe-Ala-OCH_3		0% (300)

[a] Nonstandard amino acid abbreviations are as follows: Dpr = L-diaminopropanoic acid, Ise = L-isoserine, D,L-α-MeSer = D,L-α-methylserine, Cha = cyclohexylalanine, 1Np = L-3-(1′-naphthyl)alanine, 2Np = L-3-(2′-napthyl)alanine, Bpa = L-3-(4′-(1′,1″-biphenylyl)alanine, Dpa = L-3,3-diphenylalanine, Mit = 3-iodo-L-tyrosine, Dit = 3,5-diiodo-L-tyrosine. [b] Range of IC_{50} determined. [c] Pyr = 2-pyridyl.

potency. It was hypothesized that the functionality of the carboxamide side chain was situated such that it was involved in a hydrogen bond with the enzyme or the pentapeptide itself, which was essential for inhibition of MLCK. Subsequent work which replaced Asn with nonfunctional hydrophobic amino acids Ala and Val, however, resulted in pentapeptide inhibitors equipotent with **10**.

The Val subsite was investigated less extensively than the other four amino acids. In general, the substitution for Val with hydrophobic amino acids of varying steric requirements (Met, Leu, Phe) resulted in inhibitors with similar properties to the Val analogue (**32–34**). To investigate whether Val was acting merely as a spacer element within the pentapeptide or was contributing to the observed inhibition via a hydrophobic binding contribution, two additional amino acids were investigated. Thr has a polar side chain residue which is isosteric with Val while Gly acts only as a spacer and makes no contribution from specific side-chain interactions. Both substitutions resulted in poorer inhibitors (**35**, **36**), suggesting that the Val side chain is involved in hydrophobic binding which is disrupted by the introduction of the polar hydroxyl group of Thr.

We examined the Phe position more extensively within the context of the Ser-Ala-Val-Phe-Ala-OBzl template, since we observed, upon modification of this site, a trend toward more potent inhibition. As can be seen in Table I, we observed better binding to the enzyme with more hydrophobic amino acids. Peptides incorporating the naturally occurring amino acids Trp (**37**) and Tyr (**38**) have inhibition values which bracket that observed with Phe. Tyr, which because of its hydroxyl group is more hydrophilic, had an IC_{50} of 45 μM while Trp, with a more hydrophobic side chain, was a more potent inhibitor with an IC_{50} = 6 μM. This trend is confirmed with peptides containing the synthetic hydrophobic amino acids, Cha = Cyclohexylalanine (**39**), 1Np = L-3-(1′-napthyl)alanine (**40**), 2Np = L-3-(2′-napthyl)alanine (**41**), Bpa = L-3-(4′-(1′,1″-biphenylyl)alanine (**42**), and Dpa = L-3,3-diphenylalanine (**43**), all of which are both more hydrophobic than Phe and

are inhibitors more active versus MLCK. These results suggest that there is a large hydrophobic pocket or surface on MLCK which is more fully occupied by these larger side chains, resulting in stronger hydrophobic binding forces and more potent inhibitors. Consistent with this notion is that the hydroxyl group of Tyr which slightly disrupts these hydrophobic forces can be masked by the introduction of a large iodine atom ortho to the hydroxyl group (Mit = 3-iodo-L-tyrosine), restoring inhibitory potency (**44**). The slight reduction in inhibition upon diiodination of a Tyr ring (Dit = 3,5-diiodo-L-tyrosine) suggests that there is a limit in the size of this hydrophobic pocket or surface or that a simple hydrophobic effect may not account for the total interaction (**45**).

We have stated previously that in general our inhibitors demonstrated comparable inhibition of both the native enzyme and the Ca^{2+}-calmodulin-independent enzyme. This is certainly true of pentapeptide template (**10**) and, as can be seen in Table I, the 1Np (**40**) containing peptide. The Bpa analogue (**42**) however appears to be, perhaps, an exception to this general observation, exhibiting 5-fold better inhibition toward the native enzyme than that of the Ca^{2+}-calmodulin-independent enzyme.

The C terminal alanine ester appears to be an extension of the hydrophobic substructure which also incorporates the Val and Phe residues. Studies by Kemp and coworkers have shown that in tridecapeptide substrates (MLC^{11-23}) of myosin light chain kinase the substitution of Ser for Ala at position 23 results in a modest increase in the K_m for the peptide.[5] Analogously, we observed a small increase in the IC_{50} or **46** in which Ser is substituted for Ala. These observations are consistent with a hydrophobic contribution from this site. We were particularly interested in the incremental contribution of the carboxyl protecting group to inhibition. The C terminal benzyl ester was chosen originally largely for synthetic flexibility, however the general hydrophobic nature of the C terminal residues suggested that a more detailed study was merited. Extension of the ester chain length by one or three methylene groups relative to the benzyl ester produced molecules (**47**, **48**) which exhibited only minor differences in inhibition. The benzyl amide (**49**) was also a transparent change, suggesting that the nitrogen and oxygen exhibited similar properties and that hydrogen bonding of the amide did not contribute substantially to affinity. We observed, however, a dramatic change in the 2-pyridine methyl amide capped peptide (**50**). Substitution of this end group, which is sterically identical with the benzyl amide peptide discussed above, produced a very poor inhibitor. Of course the fundamental difference between the inhibitory compounds and the pyridine methyl amide pentapeptide is reflected in the substantial hydrophobic contribution of the benzyl group when compared to the relatively hydrophilic pyridylmethyl analogue. The four remaining peptides were prepared to explore this hydrophobic effect further. Each of the substitutions of Ala-OH (**51**), the free acid, Ala-NH_2 (**52**), Ala-OMe (**53**), and Ala-O^{n}Bu (**54**) for Ala-OBzl resulted in a peptide with dramatically diminished inhibitory properties.

To assess the contributions of amino acid configurations to the inhibition of myosin light chain kinase we prepared peptide analogues (**55**–**62**, Table II) of Ser-Asn-Val-Phe-Ala-OBzl in which the stereochemistry at the α carbon of amino acid residues was changed from the naturally occurring L, to D. The absolute configuration of the Ser, Phe, and Ala residues appears to be relatively unimportant to biological activity (**55**, **58**, **59**). On the other hand, when the configuration of either the Asn or Val is inverted at the α carbon, individually (**56**, **57**) or as a pair (**61**), enzyme affinity is substantially reduced and these peptides are poor inhibitors. A most surprising result was observed when all the stereocenters were inverted to produce the enantiomer (**62**) of our prototypical inhibitory pentapeptide. *This all* D *peptide retains the full inhibitory potency of the parent pentapeptide with an all* L *stereochemistry*. We were sufficiently surprised by this result to seek additional confirmation of the chemical structure beyond the usual criteria detailed in the Experimental Section. Circular dichroism (data not shown) confirmed our assignment of the stereochemistry since the CD spectrum of the all D configuration peptide was equivalent in shape but opposite in sign to that of the all L peptide. Thus the sensitivity of inhibitors to changes at Asn and Val in peptides of mixed stereochemistry is contrasted with an apparent lack of effect in the peptide at these residues in the all D peptide. This result might suggest that it is primarily the side chains which contribute to inhibitory potency of these compounds and not the backbone atoms.[15]

Table II. Pentapeptide Analogues Containing D-Configuration Residues[a]

	peptide structure	IC_{50}, μM, or % inhibn (concn) native	IC_{50}, μM, or % inhibn (concn) independent
55	*Ser*-Asn-Val-Phe-Ala-OBzl	5	12
56	Ser-*Asn*-Val-Phe-Ala-OBzl	0% (100)	
57	Ser-Asn-*Val*-Phe-Ala-OBzl	0% (100)	
58	Ser-Asn-Val-*Phe*-Ala-OBzl		75
59	Ser-Asn-Val-Phe-*Ala*-OBzl		64
60	*Ser-Asn*-Val-Phe-Ala-OBzl	14% (100)	
61	Ser-*Asn-Val*-Phe-Ala-OBzl		186
62	*Ser-Asn-Val-Phe-Ala*-OBzl	12	

[a] D-configuration amino acids are italic.

These observations led to a systematic synthetic effort to replace the individual backbone amide and ester bonds to delineate the contribution, if any, of these bonds to inhibitory potency. As a further goal we sought to enhance metabolic stability with these changes. Our pentapeptide analogues containing pseudopeptide amide bonds (**63**–**68**) and nonpeptide mimics (**69**–**73**) of Phe-Ala-OBzl are shown in Tables III and IV, respectively. For our investigations we chose iminomethylene pseudopeptides which share certain hydrogen bond donor characteristics with amide bonds but will which generally exhibit greater conformational mobility, and of course, can exist in a positively charged protonated state. We also utilized *trans*-alkene peptide isosteres which mimic quite closely the geometric constraints imposed by a normal trans peptide bond but lack any of the hydrogen-bonding properties characteristic to amide bonds.

The replacement of the Ser-Asn and Asn-Val peptide bonds in the pentapeptide template with iminomethylene (**63**, **64**) or *trans*-alkene linkages (**66**, **67**) dramatically reduced inhibitor potency. The iminomethylene linkage between the Val-Phe residues also significantly reduced inhibitor potency (**65**), but the *trans*-alkene linkage was an effective replacement for the Val-Phe amide bond (**68**). This systematic replacement of the individual backbone amide bonds suggests that it is only the trans orientation imposed by the amide linkage between the Val-Phe resi-

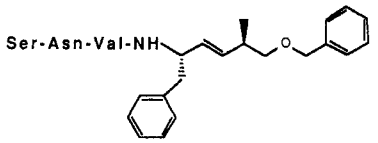
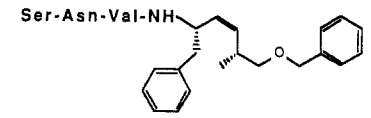
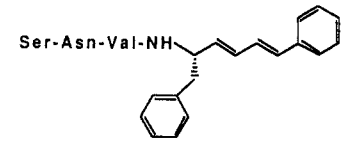
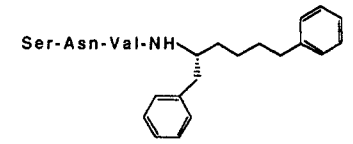
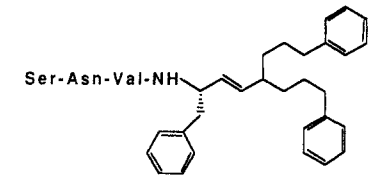
(15) Molecular modeling of these of the all D- and all L-compounds leads to a rationalization of these observed results. Flexible fitting of the two peptides indicates that atoms of the amino acid side chains of the enantiomers can be made to coincide, when the backbones are in an extended conformation, by allowing the backbone atoms to diverge from complete coincidance.

Table III. Pentapeptide Analogues Containing Pseudopeptide Amide Bonds

	peptide structure	IC_{50}, μM, or % inhibn (concn) native	independent
63	Ser-Ψ(CH_2NH)-Asn-Val-Phe-Ala-NHBzl	*a*	0% (100)
64	Ser-Asn-Ψ(CH_2NH)-Val-Phe-Ala-OBzl		33% (100)
65	Ser-Asn-Val-Ψ(CH_2NH)-Phe-Ala-OBzl		262
66	Ser-Ψ-trans(CH=CH)-Asn-Val-Phe-Ala-NHBzl		0% (100)
67	Ser-Asn-Ψ-*trans*(CH=CH)-Val-Phe-Ala-NHBzl		278
68	Ser-Asn-Val-Ψ-*trans*(CH=CH)-Phe-Ala-OBzl		36

[a] Evaluated only against calmodulin-independent MLCK.

Table IV. Pentapeptide Analogues Containing Nonpeptide Mimics of Phe-Ala-OBzl

	peptide structure	IC_{50}, μM native	imdependent
69			23
70			17
71			3.9
72			12
73			0.60

[a] Evaluated only against calmodulin-independent MLCK.

dues which is important for efficient enzyme binding. On the other hand, the results with *trans*-alkene substitutions suggest that elements of the Ser-Asn and Asn-Val amide bonds are involved in hydrogen bonds which are significant contributors to the potency of enzyme inhibitors. These may be intermolecular hydrogen bonds with the enzyme or intramolecular peptide bonds which stabilize the conformation of the inhibitor.

Our strategy to replace all the heteroatoms in the Phe-Ala-OBzl dipeptide unit did indeed prove to be fruitful. As Table IV shows, all substitutions resulted in equal or more substantially potent inhibitors (**69–73**). These efforts led to the design of compound **73** our most potent substrate-based inhibitor of MLCK, which has an IC_{50} or 0.60 μM. The results confirmed our suspicions that the heteroatoms of the Phe-Ala-OBzl backbone do not

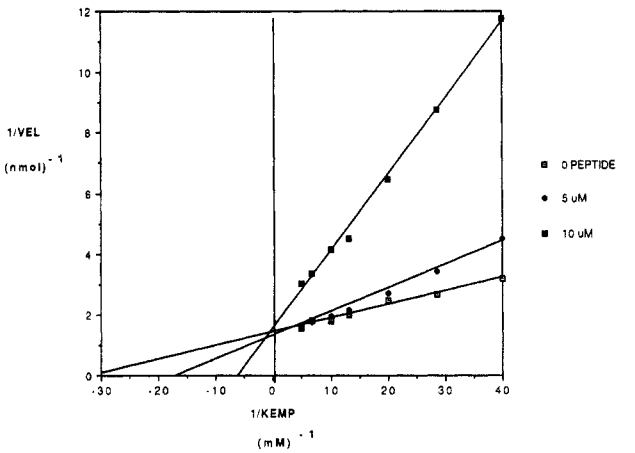


Figure 1. Lineweaver–Burk analysis of the kinetics of inhibition of 0, 5, and 10 μM Ser-Asn-Val-1Np-Ala-OBzl as a function of varying concentrations of Kemptamide. ATP concentration for this experiment was 100 μM and other experimental conditions are identical with those detailed in the Experimental Section. Plots represent a least-squares fit to the experimental data. Intersection of the lines on the 1/VEL axis is characteristic of competitive inhibition.

contribute to the potency of the inhibitors and may possibly disrupt the hydrophobic binding contribution of this portion of the molecule.

To determine whether the inhibitory peptides were themselves substrates for MLCK, the peptides were incubated for 30 min at 24 °C with calmodulin-dependent MLCK and calmodulin. Assay mixtures were fractionated on anion-exchange columns to separate unreacted ATP from the peptides. Radioactivity incorporated into the peptides was determined by scintillation counting. In order to maximize incorporation, peptides were used at 1 mM. At significantly higher concentrations, the peptides were insoluble. As shown in Table V, Calcium-dependent phosphate incorporation into the peptides **10** and **11** was extremely low. The first peptide incorporated phosphate at a rate of 0.017 pmol/min to a final total incorporation of 10^{-5} mol of P/mol of peptide, while incorporation into the second peptide was below the limits of detection. As a control, incorporation into 50 μM Kemptamide (**1**), a peptide substrate of MLCK, was measured at the same time, by using identical assay conditions. The rate of incorporation was 45 pmol/min to a final level of 0.54 mol of P/mol of peptide. The reaction was terminated before complete phosphorylation was achieved. Although limited solubility prevented the determination of a K_m or V_{max} for

Table V. Phosphorylation of Peptides by Myosin Light Chain Kinase

	peptide structure	concn, μM	rate of phosphate incorpn, pmol/min
1	Lys-Lys-Arg-Pro-Gln-Arg-Ala-Thr-Ser-Asn-Val-Phe-Ser-NH_2[a]	50	45
10	Ser-Asn-Val-Phe-Ala-OBzl	1000	0.017
11	Thr-Ser-Asn-Val-Phe-Ala-OBzl	1000	*b*

[a] Kemptamide. [b] Undetectable.

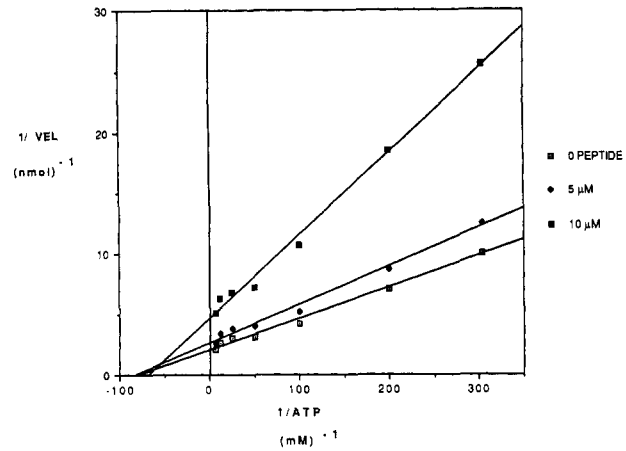


Figure 2. Lineweaver–Burk analysis of the kinetics of inhibition of 0, 5, and 10 μM Ser-Asn-Val-1Np-Ala-OBzl as a function of varying concentrations of ATP. Kemptamide concentration for this experiment was 100 μM and other experimental conditions are identical with those detailed in the Experimental Section. Plots represent a least-squares fit to the experimental data. Intersection of the lines on the 1/ATP axis is characteristic of noncompetitive inhibition.

these peptides, they were clearly very poor substrates as compared to Kemptamide.

The kinetics of the enzyme inhibition by **40**, one of the more potent pentapeptides, was studied as a function of the concentration of the two substrates. Examination of double reciprocal plots clearly indicates competitive inhibition vs the peptide substrate Kemptamide (Figure 1) and noncompetitive inhibition vs ATP (Figure 2).

Conclusions

The pentapeptide Ser-Asn-Val-Phe-Ala-OBzl is the minimum sequence of the MLC substrate that inhibits MLCK. A hydrophobic substituent at the C-terminus is essential for enzyme inhibition. Ser-Asn-Val-Phe-Ala-OBzl and structurally related peptides are not operating by calmodulin antagonism, are not competitive with ATP, and are not substrates for MLCK. The peptides are competitive with Kemptamide, a synthetic tridecapeptide substrate of MLCK.

The amino acid side chain binding requirements of the kinase are as follows: At Ser[19], a hydrophilic functional group is necessary for inhibition; at Asn[20], the methylene carboxamide side chain cannot be replaced by functionality other than short alkyl groups. The Val[21] residue can be replaced with other hydrophobic residues with retention of enzyme inhibition, but not with the hydrophilic isosteric amino acid Thr. At Phe[22], the importance of hydrophobic interactions to enzyme inhibition is further emphasized by the increased potencies of peptides in which the Phe side chain is replaced with larger hydrophobic structures. At Ala[23]-OBzl, the C-terminal Ala benzyl ester appears to be an extension of the hydrophobic substructure which also includes the Val and Phe residues. It is interesting to note that in MLC isolated from bovine and avian smooth muscle the two residues which follow Ala[23] are Met[24] and Phe[25]. The contribution of the benzyl ester is consistent with binding to this extended hydrophobic site.

The enzyme affinity is substantially reduced when the stereochemistry at the α-CH centers is inverted at the Asn or Val residues. The loss of inhibitory potency suggests that the conformation imposed by these residues on the pentapeptide is essential for efficient enzyme binding. The most startling result is that inversion of all the stereocenters, to produce the enantiomer of Ser-Asn-Val-Phe-Ala-OBzl, does not diminish enzyme inhibition.

Replacement of the Phe-Ala-OBzl dipeptide subunit with hydrophobic, nonpeptide, organic fragments results in equal or substantially more potent inhibitors. The backbone heteroatoms of this portion of the peptide do not contribute to enzyme inhibition and, if anything, disrupt the hydrophobic contribution to binding in these molecules. The results of systematic replacement of the individual backbone amide bonds in the remaining portion of the inhibitor suggest that only the trans orientation imposed by the amide linkage between the Val-Phe residues is important for efficient enzyme binding, but portions of the Ser-Asn and Asn-Val amide bonds are involved in hydrogen bonds.

Experimental Section

N$^{\alpha}$-*tert*-Butyloxycarbonyl and FMOC amino acids and benzyl esters were supplied by Bachem, Inc., Sigma Chemical Co., or Chemical Dynamics Corp. [γ-^{32}P]ATP was obtained from ICN, calmodulin was purchased from Boehringer-Mannheim, and all other chemicals were obtained from the Sigma or Aldrich Chemical Co. The nonstandard amino acids, Bpa and Dpa, were prepared according to literature procedures as previously described.[16,17] Boc amino acids were prepared as described by Tarbel et al.[18] All reactions were carried out under an inert atmosphere. ^{1}H NMR spectra were recorded on Varian XL200, XL300, or XL400 spectrometers. FAB mass spectra were recorded on a Varian MAT312. Short-path chromatography refers to the procedures described by Hunt and Rigby.[19]

Peptide Synthesis. The peptides (**7–62**) were prepared by stepwise or fragment condensation in solution and by stepwise solid-phase method on a Sasrin resin[20] by using FMOC methodology with an Applied Biosystems Model 430A peptide synthesizer. Solution couplings of the free acids and neutralized amines were carried out in the presence of 1-hydroxybenzotriazole with a water-soluble carbodiimide such as 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide (HOBT and DCI).[21] All peptides, pseudopeptides, and peptide isosteres were purified by preparative reverse-phase chromatography utilizing Rainin Dynamax C-8 or C-18 columns and an acetonitrile/water gradient buffered with 0.2% TFA. The iodinated tyrosine peptides were prepared in the standard fashion by treatment of Ser-Asn-Val-Tyr-Ala-OBzl with Chloramine T and NaI[22] and the di- and monoiodinated products were separated by HPLC chromatography. All peptides were characterized by high-field ^{1}H NMR and FAB mass spectral determinations and were homogeneous by reversed-phase chromatography on an analytical C-18 column.

Preparation of Iminomethylene Peptide Isosteres. The iminomethylene peptides were prepared by utilizing fragment condensations according to the standard methodology described by Martinez et al.[23] and references contained therein. HPLC purification gave the pseudopeptides as white amorphous powders.

Ser-Ψ(CH$_2$NH)-Asn-Val-Phe-NHBzl (63). 300-MHz ^{1}H NMR (DMSO-d_6): δ 0.68 and 0.77 (6 H, d, 2 $CH_{3\,Val}$), 1.28 (3 H, d, $CH_{3\,Ala}$), 2.00 (1 H, m, $CH(CH_3)_{2\,Val}$), 2.73–2.93 (3 H, m, $CH_{2\,Asn}$ and 1 H of $CH_{2\,Phe}$), 3.04–3.23 (3 H, m, CH_2N and 1 H of $CH_{2\,Phe}$), 3.41–3.66 (3 H, m, $CH_{2\,Ser}$ and CH_{Ser}), 4.13 (1 H, m, NCH_{Val}), 4.42–4.73 (4 H, m, $CH_{2\,Bzl}$, CH_{Asn}, and CH_{Ala}), 4.52 (1 H, m, CH_{Phe}), 7.16–7.35 (11 H, m, 2 Ph and 1 H of $CONH_{2\,Asn}$), 7.3 (1 H, s,

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$CONH_{2\,Asn}$), 7.79, 8.12, 8.35–8.61, 8.71, 9.30–9.70, 9.75–10.10 (9 H, m, $NH_{backbone}$), 8.26 (1 H, t, OH). FAB mass spectrum: $(M + 1)^+ = 612$ *m/e*.

Ser-Asn-Ψ(CH_2NH)-Val-Phe-NHBzl (64). 200-MHz ^1H NMR (DMSO-d_6): δ 0.88 and 0.97 (6 H, d, 2 $CH_{3\,Val}$), 1.35 (3 H, d, $CH_{3\,Ala}$), 2.14–2.42 (3 H, m, $CH(CH_3)_{2\,Val}$ and $CH_{2\,Asn}$), 2.63–2.90 and 3.01–3.18 (4 H, m, CH_2N and $CH_{2\,Phe}$), 3.54–3.84 (4 H, m, NCH_{Val}, $CH_{2\,Ser}$, and CH_{Ser}), 4.17 (1 H, m, CH_{Asn}), 4.35 (1 H, m, CH_{Ala}), 4.77 (1 H, m, CH_{Phe}), 5.13 (2 H, s, $CH_{2\,Bzl}$), 5.48 (1 H, t, OH), 7.10 and 7.63 (2 H, s, $CONH_{2\,Asn}$), 7.28 (5 H, s, Ph_{Bzl}), 7.39 (5 H, s, Ph_{Phe}), 8.19–8.35 and 8.75–9.14 (8 H, m, $NH_{backbone}$). FAB mass spectrum: $(M + 1)^+ = 612$ *m/e*.

Ser-Asn-Val-Ψ(CH_2NH)-Phe-NHBzl (65). 200-MHz ^1H NMR (DMSO-d_6): δ 0.77 (6 H, d, 2 $CH_{3\,Val}$), 1.32 (3 H, d, $CH_{3\,Ala}$), 1.67 (1 H, m, $CH(CH_3)_{2\,Val}$), 2.43–2.75 (2 H, m, $CH_{2\,Asn}$), 2.83–3.25 (4 H, m, CH_2N and $CH_{2\,Phe}$), 3.55–3.94 (4 H, m, NCH_{Val}, $CH_{2\,Ser}$, and CH_{Ser}), 3.96–4.17 (1 H, m, CH_{Phe}), 4.34 (1 H, m, CH_{Ala}), 4.59 (1 H, m, CH_{Asn}), 5.10 (2 H, s, $CH_{2\,Bzl}$), 5.53 (1 H, t, OH), 7.18 and 7.40 (2 H, s, $CONH_{2\,Asn}$), 7.25 (5 H, s, Ph_{Bzl}), 7.34 (5 H, s, Ph_{Phe}), 7.80, 8.06, 8.27, 8.82, 9.14, 9.33 (8 H, m, $NH_{backbone}$). FAB mass spectrum: $(M + 1)^+ = 612$ *m/e*.

Preparation of *trans*-Alkene Peptide Isosteres. The protected *trans*-alkene isosteres of the dipeptides Cbz-Ser-*trans*(CH=CH)-Asn, Cbz-Asn-*trans*(CH=CH)-Val, and BOC-Val-*trans*(CH=CN)-Phe were prepared according to the procedures of Lehman de Gaeta et al.[24] and Spaltenstein et al.[25] Each was then coupled with the appropriate peptide fragments and deprotected by utilizing standard solution-phase peptide-synthesis techniques. HPLC purification gave each pseudopeptide as a homogeneous white amorphous powder.

Ser-*trans*(CH=CH)-Asn-Val-Phe-NHBzl (66). 400-MHz ^1H NMR (CD_3OD): δ 0.72–0.84 (6 H, m, 2 $CH_{3\,Val}$), 1.27 (3 H, d, $CH_{3\,Ala}$), 1.92 (1 H, m, $CH(CH_3)_{2\,Val}$), 2.37 and 2.62 (2 H, dd, $CH_{2\,Asn}$), 2.84 and 3.05 (2 H, dd, $CH_{2\,Phe}$), 3.38–3.70 (4 H, m, $CH_{2\,Ser}$, CH_{Ser}, and CH_{Asn}), 4.01 (1 H, m, NCH_{Val}), 4.21–4.29 (3 H, m, $CH_{2\,Bzl}$ and CH_{Ala}), 4.55 (1 H, m, CH_{Phe}), 5.60 and 5.81 (2 H, dd, CH=CH, J = 14 Hz), 7.07–7.26 (10 H, m, 2 Ph), 7.83, 7.92, 8.04, 8.09 (~4 H, m, NH). FAB mass spectrum: $(M + 1)^+ = 610$ *m/e*.

Ser-Asn-*trans*(CH=CH)-Val-Phe-NHBzl (67). 200-MHz ^1H NMR (DMSO-d_6): δ 0.68–0.81 (6 H, m, 2 $CH_{3\,Val}$), 1.24 (3 H, d, $CH_{3\,Ala}$), 1.83 (1 H, m, $CH(CH_3)_{2\,Val}$), 2.27 (2 H, m, $CH_{2\,Asn}$), 2.71–3.07 (3 H, m, $CH_{2\,Phe}$ and NCH_{Val}), 3.85 (2 H, m, $CH_{2\,Ser}$), 4.22–4.34 (3 H, m, CH_{Ser} and $CH_{2\,Bzl}$), 4.41–4.57 (3 H, m, CH_{Asn}, CH_{Ala}, and CH_{Phe}), 5.53–5.46 (3 H, m, OH and CH=CH, J = 12 Hz), 6.87 and 7.40 (2 H, s, $CONH_{2\,Asn}$), 7.12–7.34 (10 H, m, 2 Ph), 7.75–8.00, 8.32, 8.48 (7 H, m, $NH_{backbone}$). FAB mass spectrum: $(M + 1)^+ = 610$ *m/e*.

Ser-Asn-Val-*trans*(CH=CH)-Phe-NHBzl (68). 200-MHz ^1H NMR (DMSO-d_6): δ 0.62–0.76 (6 H, m, 2 $CH_{3\,Val}$), 1.14–1.32 (3 H, m, $CH_{3\,Ala}$), 1.41–1.68 (1 H, m, $CH(CH_3)_{2\,Val}$), 2.42–2.70 (2 H, m, $CH_{2\,Asn}$), 2.78–3.26 (3 H, m, $CH_{2\,Phe}$ and CH_{Phe}), 3.67–4.03 (4 H, m, $CH_{2\,Ser}$, CH_{Ser}, and NCH_{Val}), 4.29 (1 H, m, CH_{Ala}), 4.66 (1 H, m, CH_{Asn}), 5.09 (2 H, s, $CH_{2\,Bzl}$), 5.34 and 5.44 (2 H, dd, CH=CH, J = 14 Hz), 5.53 (1 H, t, OH), 6.96 (1 H, s, 1 H of $CONH_{2\,Asn}$), 7.09–7.27 (5 H, m, Ph_{Bzl}), 7.32–7.46 (6 H, m, Ph_{Phe} and 1 H of $CONH_{2\,Asn}$), 7.74, 8.02–8.43, 8.68 (6 H, m, $NH_{backbone}$). FAB mass spectrum: $(M + 1)^+ = 610$ *m/e*.

Preparation of the Peptides Incorporating Phe-Ala-OBzl Surrogates. Compounds **69** and **70** were prepared as follows: Methyl 3-hydroxy-2(*R*)-methylpropionate (10 g, 85 mmol, Aldrich) was stirred with 35.6 g (423 mmol) of dihydropyran and 0.16 g (0.85 mmol) of *p*-toluenesulfonic acid in 300 mL of dichloromethane for 2 h at room temperature. The reaction mixture was washed sequentially with water, saturated $NaHCO_3$, and brine. The solution was dried with anhydrous $NaSO_4$ and evaporated to give 22 g of the crude THP ether which was used without further purification or characterization. The crude THP ether (10 g, 49 mmol) was dissolved in anhydrous ethyl ether, cooled to 0 °C, and treated with 2.3 g (59 mmol) of lithium aluminum hydride, added carefully in small portions. After 2 h an additional 1.0 g (26 mmol) was added; the mixture was allowed to warm to room temperature and was stirred an additional 14 h. Excess hydride was carefully decomposed with an aqueous solution of $KHSO_4$. The mixture was extracted with ethyl ether; the organic layer was washed with saturated $NaHCO_3$ and brine. The aqueous layers were reextracted with dichloromethane and the combined organic layers were dried with anhydrous Na_2SO_4 and evaporated. The crude product was purified by short-path chromatography on silica eluting with CH_2Cl_2/CH_3OH (98:2) to give the 5.4 g (63%) of the desired alcohol ether isolated as an oil. 200-MHz ^1H NMR ($CDCl_3$): δ 0.91 (3 H, m, CH_3), 1.48–1.88 (6 H, m, $(CH_2)_3$), 2.05 (1 H, m, CH), 2.60 (1 H, m, OH), 3.31–3.93 (6 H, m, 3 CH_2O), 4.60 (1 H, m, OCHO).

NaH (1.4 g of a 60% suspension in mineral oil, 35 mmol) was washed successively with three aliquots (50 mL) of dry hexanes. To the flask was added 60 mL of anhydrous tetrahydrofuran and 5.35 g (31 mmol) of the above alcohol ether and the mixture was stirred at room temperature for 15 min. Benzyl bromide (6.8 g, 40 mmol) was added and the reaction stirred for 14 h. The reaction mixture was diluted with 200 mL of ethyl ether and excess sodium hydride was quenched with an aqueous solution of $KHSO_4$. The ether was washed with saturated sodium bicarbonate and brine and dried with anhydrous magnesium sulfate. The crude product was purified by short-path chromatography on silica gel eluting with hexanes/Et_2O (95:5). The product (6.3 g, 77%) was obtained as an oil. 200-MHz ^1H NMR ($CDCl_3$): δ 1.00 (3 H, d, CH_3), 1.44–1.94 (6 H, m, $-(CH_2)_3-$), 1.98–2.22 (1 H, m, CH), 3.28–3.92 (6 H, m, CH_2O), 4.52 (3 H, s, $CH_2C_6H_5$), 4.58 (1 H, b s, OCHO), 7.34 (5 H, m, C_6H_5).

Conversion of the THP ether to the bromide followed the procedure of Sonnet.[26] In 100 mL of dichloromethane, 6.85 g (26 mmol) of triphenylphosphine was dissolved and treated dropwise with bromine until and orange color persisted. The THP ether (6.28 g, 24 mmol) was added to the mixture in a single portion. After stirring for 20 min at room temperature, 50 mL of water was added, and the layers were separated. The organic layer was washed with water, dried with sodium sulfate, and concentrated to a solid. The solid was triturated with 100 mL of hexanes and the triphenylphosphine oxide precipitate was filtered and washed 3× with 50 mol of hexanes. The hexanes were concentrated under reduced pressure. The residue was purified by short-path chromatography on silica (hexane/ether, 98:2). Isolation of the desired fractions gave 5.35 g of 3-(benzyloxy)-1-bromo-2(*S*)-methylpropane (92%) as a clear oil. 200-MHz ^1H NMR ($CDCl_3$): δ 1.06 (3 H, d, CH_3), 2.26 (1 H, m, CH), 3.42–3.56 (4 H, m, CH_2OBzl and $BrCH_2$), 4.55 (2 H, s, $OCH_{2\,Bzl}$), 7.37 (5 H, s, Ph).

The bromide (5.35 g, 22 mmol) was dissolved in 50 mL of tetrahydrofuran and treated with 23 g (88 mmol) of triphenylphosphine. The reaction was refluxed for 8 days and then cooled to 0 °C. The white precipitate which formed was filtered and washed with ethyl ether to give 5.3 g (48%) of the triphenylphosphonium bromide which was used without further purification. 200-MHz ^1H NMR ($CDCl_3$): δ 0.92 (3 H, d, CH_3), 2.17 (1 H, m, CH), 3.43–4.07 (4 H, m, CH_2OBzl and CH_2P), 4.38 (2 H, m, $OCH_{2\,Bzl}$) 7.39 (5 H, m, Ph), 7.60–7.94 (15 H, m, Ph_3P).

To the vacuum-dried phosphonium salt (5.36 g, 10.6 mmol) in 15 mL of tetrahydrofuran was added 1.19 g (10.6 mmol) of potassium *tert*-butoxide and 0.035 g (0.09 mmol) of dicyclohexano-18-crown-6. *N*-(*tert*-butyloxycarbonyl)phenylalaninal[27] (2.65 g, 10.6 mmol) was dissolved in 20 mL of tetrahydrofuran and added to the reaction mixture. After stirring for 1 h at room temperature, the potassium bromide was filtered from the reaction through a plug of silica gel and the solvent was removed in vacuo. Short-path chromatography on silica (hexane/ether, 3:1) separated the cis (1.14 g) and trans (0.22 g) alkenes as oils in 33% overall yield (cis, R_f = 0.20; trans, R_f = 0.17, silica, hexane/ether, 9:1). 400-MHz ^1H NMR trans isomer ($CDCl_3$): δ 0.98 (3 H, d, CH_3), 1.39 (9 H, s, BOC), 2.44 (1 H, m, $CHCH_3$), 2.80 (2 H, m, CCH_2Ph), 3.24 (2 H, m, CH_2OBzl), 4.29–4.50 (4 H, m, NCH, NH, $OCH_{2\,Bzl}$), 5.22 (2 H, m, CH=CH, J = 15 Hz), 7.10–7.35 (10 H, m, 2 Ph).

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400-MHz ^{1}H NMR cis isomer ($CDCl_3$): δ 0.75 (3 H, d, CH_3), 1.40 (9 H, s, BOC), 2.71 (2 H, m, CCH_2Ph), 2.95 (1 H, m, $CHCH_3$), 3.24 (2 H, m, CH_2OBzl), 4.37 (1 H, m, NCH), 4.47 (2 H, s, $OCH_{2\,Bzl}$), 4.58 (1 H, m, NH), 5.24 (2 H, m, CH=CH, J = 5 Hz), 7.10–7.35 (10 H, m, 2 Ph).

With standard peptide synthesis techniques, the BOC protecting groups were removed from each isomer separately, the amines were coupled to BOC-Ser-Asn-Val, and each was then deblocked. HPLC purification gave the desired targets.

Compound **69**: 200-MHz ^{1}H NMR (DMSO-d_6): δ 0.74 (6 H, m, $CH_{3\,Val}$), 0.91 (3 H, d, $CHCH_3$), 1.96 (1 H, m, $CH(CH_3)_{2\,Val}$), 2.27–2.59 (3 H, m, $CH_{2\,Asn}$ and $CHCH_3$), 2.73 (2 H, m, CCH_2Ph), 3.18–3.35 (2 H, m, CH_2OBzl), 3.56–3.93 (3 H, m, $CH_{2\,Ser}$ and CH_{Ser}), 4.07 (1 H, m, CH_{Val}), 4.42 (3 H, s, $CH_{2\,Bzl}$ and $NCHCH$=), 4.70 (1 H, m, CH_{Asn}), 5.38–5.49 (3 H, m, OH and CH=CH, J = 14 Hz), 6.98 and 7.34 (2 H, s, $CONH_{2\,Asn}$), 7.10–7.32 (10 H, m, 2 Ph), 7.51, 7.72, 7.92–8.39, 8.78 (6 H, m, $NH_{backbone}$). FAB mass spectrum: $(M + 1)^+$ = 596 m/e.

Compound **70**: 200-MHz ^{1}H NMR (DMSO-d_6): δ 0.53 (3 H, d, $CHCH_3$), 0.86 (6 H, m, 2 $CH_{3\,Val}$), 1.97 (1 H, m, $CH(CH_3)_{2\,Val}$), 2.41–2.71 (4 H, m, $CH_{2\,Asn}$ and CCH_2Ph), 2.90 (1 H, m, $CHCH_3$), 3.09–3.32 (2 H, m, CH_2OBzl), 3.65–3.97 (3 H, m, $CH_{2\,Ser}$ and CH_{Ser}), 4.11 (1 H, m, CH_{Val}), 4.41 (2 H, s, $CH_{2\,Bzl}$), 4.76 (2 H, m, CH_{Asn}, and $NCHCH$=), 5.10–5.37 (2 H, m, CH=CH, J = 5 Hz), 5.53 (1 H, t, OH), 7.04 and 7.53 (2 H, s, $CONH_{2\,Asn}$), 7.10–7.35 (10 H, m, 2 Ph), 7.81, 8.12–8.29, 8.82 (6 H, m, $NH_{backbone}$). FAB mass spectrum: $(M + 1)^+$ = 596 m/e.

Compound **71** was prepared as follows: 2(*S*)-[(*tert*-Butoxycarbonyl)amino]-3-phenyl-1-(phenylsulfonyl)propane was prepared and coupled with cinnamaldehyde by using a modification to the methodology described by Hopkins and co-workers.[25] The sulfone (1.5 g, 4 mmol) was suspended in 16 mL of anhydrous tetrahydrofuran. The reaction was cooled to −78 °C and 2.85 mL (4 mmol) of methyl lithium solution (1.4 M in ethyl ether) was added over 3 min. After 20 min the reaction was warmed to 0 °C and stirred for 1 h. A second equivalent of methyl lithium (2.85 mL) was added and the stirring was continued for 20 min at 0 °C. The solution was then cooled again to −78 °C, 1.32 g (10 mmol) of cinnamaldehyde was added, and stirring was continued for 14 h at −78 °C. The reaction was quenched with 5 mL of saturated aqueous ammonium chloride and extracted with ethyl ether, and the ether was dried with anhydrous sodium sulfate and concentrated under reduced pressure to give the crude hydroxy sulfone, which was used without further purification or characterization.

The hydroxy sulfone was dissolved in 150 mL of methanol and cooled to 0 °C. Na_2HPO_4 (2.5 g, 15 mmol) was added followed by 25 g (50 mmol) of 5% Na(Hg) and the reaction was stirred for 5 h at 0 °C. The reaction was diluted with water and extracted with dichloromethane; the organic layer was dried with anhydrous sodium sulfate and concentrated. Short-path chromatography (hexane/ether, 3:1) gave 0.56 g (40%) of *trans*-2(*S*)-[(*tert*-butoxycarbonyl)amino]-1,6-diphenyl-3,5-hexadiene as an oil. 200-MHz ^{1}H NMR ($CDCl_3$): δ 1.44 (9 H, s, BOC), 2.89 (2 H, m, CH_2), 4.49 (1 H, m, NCH), 4.88 (1 H, m, NH), 5.31, 5.74, 6.11–6.97 (4 H, m, CH=CHCH=CH, J = 15.5 Hz), 7.15–7.39 (10 H, m, 2 Ph). CI mass spectrum: $(M + 1)^+$ = 350 m/e.

The diene was deprotected and coupled to BOC-Ser-Ala-Val which, after complete deprotection and purification by reversed-phase HPLC, gave **71** as a white, amorphous solid. 200-MHz ^{1}H NMR ($CDCl_3$): δ 0.73 (6 H, m, 2 $CH_{3\,Val}$), 1.99 (1 H, m, $CH(CH_3)_{2\,Val}$), 2.42–2.69 (2 H, m, $CH_{2\,Asn}$), 2.81 (2 H, m, CH_2Ph), 3.64–3.89 (3 H, m, $CH_{2\,Ser}$ and CH_{Ser}), 4.08 (1 H, m, NCH_{Val}), 4.59 (1 H, m, $NCHCH$=), 4.72 (1 H, m, CH_{Asn}), 5.49 (1 H, m, OH), 5.82, 6.49, 6.6, 6.85 (4 H, m, CH=CHCH=CH, J = 15 Hz), 7.04 and 7.55 (2 H, s, $CONH_{2\,Asn}$), 7.15–7.38 (10 H, m, 2 Ph), 7.82, 8.10–8.28, 8.80 (6 H, m, $NH_{backbone}$). FAB mass spectrum: $(M + 1)^+$ = 550 m/e.

Preparation of 72. *trans*-2(*S*)-[(*tert*-Butoxycarbonyl)amino]-1,6-diphenyl-3,5-hexadiene (0.30 g) was dissolved in 30 mL of ethyl acetate and hydrogenated at 60 psi with 10% Pd/C (0.30 g) to give a quantitative yield of 2(*S*)-[(*tert*-butoxycarbonyl)amino]-1,6-diphenylhexane which required no purification. 200-MHz ^{1}H NMR ($CDCl_3$): δ 0.83–1.71 (15 H, m, BOC and $(CH_2)_3Bzl$), 2.57 (2 H, t, CH_2CH_2Ph), 2.73 (2 H, d, $CHCH_2Ph$), 3.78 (1 H, m, NCH), 4.29 (1 H, m, NH), 7.11–7.33 (10 H, m, 2 Ph). FAB mass spectrum: $(M + 1)^+$ = 354 m/e.

The hydrogenated product was deblocked, coupled with BOC-Ser-Asn-Val, and deprotected by using standard peptide synthesis techniques. Compound **72** was isolated as a white powder after HPLC chromatography. 200-MHz ^{1}H NMR ($CDCl_3$): δ 0.75 (6 H, m, 2 $CH_{3\,Val}$), 1.16–1.57 (6 H, m, $(CH_2)_3Bzl$), 1.99 (1 H, m, $CH(CH_3)_{2\,Val}$), 2.42–2.72 (6 H, m, $CH_{2\,Asn}$ and 2 CH_2Ph), 3.59–3.95 (4 H, m, $CH_{2\,Ser}$, $NCHBzl$, and CH_{Ser}), 4.06 (1 H, m, NCH_{Val}), 4.71 (1 H, m, CH_{Asn}), 5.51 (1 H, m, OH), 7.00 and 7.59 (2 H, s, $CONH_{2\,Asn}$), 7.08–7.30 (10 H, m, 2 Ph), 7.73, 7.82, 7.98–8.33, 8.82 (6 H, m, $NH_{backbone}$). FAB mass spectrum: $(M + 1)^+$ = 554 m/e.

Preparation of 73. Trimethylsulfoxonium iodide (2.28 g, 10.8 mmol) was dissolved in 30 mL of dry DMSO and treated with 4.10 mL (10.2 mmol) of *n*-butyllithium (2.5 M in hexane) for 30 min. To the reaction mixture was added 2.45 g (9.20 mmol) of 1,7-diphenylheptan-4-one[28] dissolved in 5 mL of DMSO and the mixture was stirred at room temperature for 14 h. The reaction mixture was poured into 100 mL of ice water and extracted with ethyl ether. The ethyl layer was washed with brine and dried with anhydrous magnesium sulfate and concentrated to 2.2 g of an oil. Partial purification of the epoxide was effected, with some difficulty, on two successive short-path chromatography columns (silica, hexane/acetone 9:1 then hexane/ethyl ether 95:5). The product was used without additional purification. 200-MHz ^{1}H NMR (DMSO-d_6): δ 1.51–1.82 (8 H, m), 2.52–2.74 (6 H, m), 7.09–7.35 (10 H, m). CI mass spectrum: $(M + 1)^+$ = 281, $(M + NH_4)^+$ = 298 m/e.

The crude epoxide (0.72 g, 2.5 mmol) was dissolved in 55 mL of freshly distilled benzene and cooled to 10 °C. To this was added 1.45 mL (11.8 mmol) of BF_3/Et_2O; after 30 min an additional 1.0 mL (8.2 mmol) of BF_3/Et_2O was added, the temperature was allowed to rise to room temperature, and the stirring was continued for 14 h. The reaction mixture was partitioned between 100 mL each of ethyl ether and saturated potassium carbonate solution. The organic layer was washed with brine and dried with anhydrous sodium sulfate. Concentration under reduced pressure gave 0.70 g of crude 5-phenyl-2-(3-phenylpropyl)pentan-1-al, which was used without further purification. 200-MHz ^{1}H NMR ($CDCl_3$): δ 1.58 (8 H, m, 2 $(CH_2)_2Bzl$), 2.56–2.72 (5 H, m, $CHOCH$ and 2 CH_2Ph), 7.11–7.32 (10 H, m, 2 Ph), 9.54 (1 H, d, CHO).

This *trans*-alkene was prepared by the condensation of 2-(*S*)-[(*tert*-butoxycarbonyl)amino]-3-phenyl-1-(phenylsulfonyl)-propane[25] with 5-phenyl-2-(3-phenylpropyl)pentan-1-al under identical conditions to those utilized in condensation with cinnamaldehyde to produce the *trans*-alkene intermediate in the synthesis of **71**. *trans*-2(*S*)-[(tert-Butoxycarbonyl)amino]-1,8-diphenyl-5-(3-phenylpropyl)-3-octene was obtained as an oil (28% overall yield, two steps) after short-path chromatography (hexane/ether, 1:1). 200-MHz ^{1}H NMR ($CDCl_3$): δ 1.22–1.65 (17 H, m, BOC and 2 $(CH_2)_2Bzl$), 2.52 (4 H, m, CH_2CH_2Ph), 2.78 (2 H, m, $CHCH_2Ph$), 3.51 (1 H, m, $=CHCH(CH_2)_2$), 4.37 (2 H, m, NCH and NH), 5.17 (2 H, m, CH=CH, J = 14 Hz), 7.07–7.30 (15 H, m, 3 Ph).

Deblocking this BOC-alkene, coupling with BOC-Ser-Asn-Val, and deprotecting using standard methodologies gave **73** as white solid after HPLC purification. 400-MHz ^{1}H NMR (CD_3OD): δ 0.70 (6 H, m, 2 $CH_{3\,Val}$), 0.98–1.52 (8 H, m, 2 $(CH_2)_2Bzl$), 1.87 (1 H, m, $CH(CH_3)_{2\,Val}$), 2.36–2.81 (8 H, m, $CH_{2\,Asn}$ and 3 CH_2Ph), 3.25 (1 H, m, $=CHCH(CH_2)_2$), 3.74 (2 H, m, $CH_{2\,Ser}$), 3.85 (1 H, m, CH_{Ser}), 4.10 (1 H, m, NCH_{Val}), 4.42 (1 H, m, $NCHCH$=), 4.78 (1 H, m, CH_{Asn}), 5.08 and 5.29 (2 H, m, CH=CH, J = 17.5 Hz), 6.94 and 7.45 (2 H, s, $CONH_{2\,Asn}$), 7.05–7.29 (15 H, m, 3 Ph), 7.65, 8.12, 8.75 (~6 H, m, $NH_{backbone}$). FAB mass spectrum: $(M + 1)^+$ = 698 m/e.

Protein Preparations. Calmodulin-dependent chicken gizzard or bovine aorta MLCK or greater than 95% homogeneity prepared with the method of Ngai et al.[29] was generously provided by Dr. Paul Trotta, Schering-Plough Research. Calmodulin-independent MLCK was prepared by modifications of the digestion procedure of Tanaka et al.,[30] as described by Foster et al.[31] This preparation

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of calmodulin-independent MLCK is stable for over 1 year at -70 °C.

Enzyme Assays. MLCK was assayed with the synthetic peptide substrate Kemptamide, a synthetic tridecapeptide with a sequence corresponding to residues 11-23 of gizzard myosin light chain except for a carboxy-terminal serine-NH_2.[5] The standard assay was performed in a total volume of 100 μL containing 20 mM Tris-HCl, pH 7.2, 10 μM $MgCl_2$, 100 μM [γ-^{32}P]ATP with a specific activity of 300-1000 cpm/pmol, 50 μM Kemptamide, 0.02 μg of enzyme. When calcium-dependent activity was measured, calmodulin was added at 200 nM, and assays contained 1 mM EGTA or 1 mM EGTA plus approximately 200 μM excess of free calcium. Calcium-independent activity was negligible in the calcium-dependent gizzard enzyme. Assays were initiated by addition of ATP and stopped by addition of 200 μM HCl. Phosphate incorporated into the basic substrate was measured by spotting an aliquot on phosphocellulose filter paper, washing, and counting the papers.

For determination of IC_{50} values, inhibitors were tested in duplicate at four concentrations, including concentrations above and below the IC_{50}, and values were determined graphically. Values are reported as the mean of measured IC_{50}'s or a percent inhibition at a concentration when the IC_{50} was not determined. K_i values were determined for key inhibitors by secondary plots of apparent K_m vs inhibitor concentration.

In order to determine whether the inhibitory peptides were phosphorylated by MLCK, a column anion-exchange method procedure was used.[32] Calmodulin-dependent chicken gizzard MLCK was used for this assay, and assay mixtures were as described above, except that 1 mM peptide was used in place of Kemptamide.

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Registry No. **1**, 89315-28-6; **7**, 73393-25-6; **8**, 124318-95-2; **9**, 124318-96-3; **10**, 124318-97-4; **11**, 124340-25-6; **12**, 124318-98-5; **13**, 124318-99-6; **14**, 124319-00-2; **15**, 124319-01-3; **16**, 124319-02-4; **17**, 124319-03-5; **18**, 124319-04-6; **19**, 124319-05-7; **20**, 124319-06-8; **21**, 124319-07-9; **22**, 124319-08-0; **23**, 124319-09-1; **24**, 124319-10-4; **25**, 124319-11-5; **26**, 124340-26-7; **27**, 124319-12-6; **28**, 124319-13-7; **29**, 124319-14-8; **30**, 124319-15-9; **31**, 124319-16-0; **32**, 124319-17-1; **33**, 124319-18-2; **34**, 124319-19-3; **35**, 124319-20-6; **36**, 124319-21-7; **37**, 124319-22-8; **38**, 124319-23-9; **39**, 124377-86-2; **40**, 124377-87-3; **41**, 124378-71-8; **42**, 124377-88-4; **43**, 124377-89-5; **44**, 124319-24-0; **45**, 124319-25-1; **46**, 124319-26-2; **47**, 124340-27-8; **48**, 124340-28-9; **49**, 124340-29-0; **50**, 124319-27-3; **51**, 124319-28-4; **52**, 124319-29-5; **53**, 124319-30-8; **54**, 124319-31-9; **55**, 124340-24-5; **56**, 124319-32-0; **57**, 124319-33-1; **58**, 124377-90-8; **59**, 124377-91-9; **60**, 124319-34-2; **61**, 124319-35-3; **62**, 124319-36-4; **63**, 124319-37-5; **64**, 124319-38-6; **65**, 124319-39-7; **66**, 124319-40-0; **67**, 124319-41-1; **68**, 124340-30-3; **69**, 124319-42-2; **70**, 124377-92-0; **71**, 124319-43-3; **72**, 124319-44-4; **73**, 124340-31-4; MLCK, 51845-53-5; (*R*)-$HOCH_2CHMeCOOMe$, 72657-23-9; (THP)OCH_2-(*R*)-CHMeCOOMe, 88557-53-3; (THP)OCH_2-(*S*)-$CHMeCH_2OH$, 88588-59-4; $PhCH_2Br$, 100-39-0; (THP)OCH_2-(*S*)-$CHMeCH_2OBzl$, 104265-23-8; (*S*)-$BrCH_2CHMeCH_2OBzl$, 63930-50-7; (*S*)-Ph_3P^+-$CH_2CHMeCH_2OBzl$·Br^-, 124340-32-5; BOC-Phe-H, 72155-45-4; (BOC)NH-(*S*)-$CH(CH_2Ph)$-(*E*)-CH=CH-(*R*)-$CHMeCH_2OBzl$, 124319-45-5; (BOC)NH-(*S*)-$CH(CH_2Ph)$-(*Z*)-CH=CH-(*R*)-$CHMeCH_2OBzl$, 124319-46-6; BOC-Ser-Asn-Val-OH, 124319-47-7; (*S*)-(BOC)$NHCH(CH_2Ph)CH_2SO_2Ph$, 108385-55-3; (*E*)-PhCH=CHCHO, 14371-10-9; (BOC)$NHCH(CH_2Ph)CH(SO_2Ph)CH$-(OH)CH=CHPh, 124319-48-8; (2*S*,3*E*,5*E*)-(BOC)NHCH-(CH_2Ph)CH=CHCH=CHPh, 124319-49-9; (*R*)-(BOC)NHCH-$(CH_2Ph)(CH_2)_4Ph$, 124340-33-6; $Ph(CH_2)_3CO(CH_2)_3Ph$, 63434-46-8; $OHCCH[(CH_2)_3Ph]_2$, 124319-51-3; (2*S*,3*E*)-(BOC)NHCH-$(CH_2Ph)CH$=$CHCH[(CH_2)_3Ph]_2$, 124319-52-4; 3,4-dihydro-2*H*-pyran, 110-87-2; 2,2-bis(3-phenylpropyl)oxirane, 124319-50-2.

Plant Antitumor Agents. 29.[1] Synthesis and Biological Activity of Ring D and Ring E Modified Analogues of Camptothecin

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The total synthesis of the pentacyclic camptothecin analogues **3** and **4** in 11 steps from *p*-tolualdehyde is described. The overall shape of compound **3** is the same as that of potent, naturally occurring camptothecin (**1a**). Despite the near spatial identity of **3** and **1b** (racemic, (20*RS*)-camptothecin) from a three-dimensional standpoint, the 9KB and 9PS cytotoxicity assays indicate at least a 40-60-fold decrease in activity of **3** compared to that of **1b**, and the isomer **4** was inactive. Similarly, studies of the inhibition of topoisomerase I activity indicated only slight activity for **3** and no activity for **4**. It is evident that the pyridone ring D is essential for antitumor activity. Three E ring modified analogues of camptothecin, **2d-f**, are described in which the net change is replacement of O by N in ring E. Compared to (20*S*)-camptothecin (**1a**) or (20*RS*)-camptothecin (**1b**), the ring E modified analogues **2d-f** display little or no cytotoxic activity, greatly reduced effect on the inhibition of topoisomerase I, and total loss of life prolongation in the in vivo L-1210 mouse leukemia assay, indicative of the highly restricted structural and electronic requirements of ring E for biological activity in camptothecin.

The discovery of the natural product (20*S*)-camptothecin (**1a**) and its potent antitumor activity more than 20 years ago[2] has led to considerable research efforts to provide a derivative or analogue suitable for use as a clinically valuable anticancer agent. In the process, considerable insight into structural requirements for antitumor activity has been gained.[3] Recently the findings that

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