Stabilized Analogs of Thymopentin. 1. 4,5-Ketomethylene Pseudopeptides

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The pentapeptide, thymopentin (Arg[1]-Lys[2]-Asp[3]-Val[4]-Tyr[5]) is known for its activity as an immunomodulating drug, but with limited half-life in plasma. In this first paper of a series of three studies, the synthesis of analogs stabilized at the peptide bond between the C-terminal amino acids via insertion of a ketomethylene moiety is described. N-Blocked pseudopeptides containing Val(k)Phe, Ala(k)Phe, and Val(k)Val units were prepared and attached to chloromethyl Merrifield resin via the carboxy terminal. Removal of the *N*-BOC group by trifluoroacetic acid was followed by sequential coupling with *N*-BOC dipeptides of aspartic acid to yield resin-bound *N*-BOC pseudotetrapeptides. Removal of *N*-BOC and coupling with *N*-BOC-*r*-*N*-tosylarginine followed by total cleavage of blocking groups and resin by HF afforded the target pseudopentapeptides. The analogs were found to compete favorably with thymopentin for binding to CEM cells, but binding was reduced by about 20−30% on average. All analogs showed significant enhancement of half-life *versus* thymopentin in mouse serum, but most showed only modest improvement in human serum. Insertion of proline or norleucine at position 2 in the chain caused a substantial increase in half-life (3−4-fold), while *N*-methylnorleucine conferred complete stability in the analogs.

In 1975, Goldstein and co-workers[1,2] reported the isolation of a 49 amino acid polypeptide from human thymus tissue. This peptide, named thymopoetin, was found to enhance *in vitro* the differentiation of prothymocytes to thymocytes among other immune-related properties such as late stage differentiation of B-cells, induction of complement receptor, and lymphoid cell transcription. Effects measured *in vivo* included induction of prothymocytes, enhancement of T-cell-dependent antibody responses, and delay of onset of autoimmune hemolytic anemia in mice. Further investigation by this group[3] found that a pentapeptide, Arg-Lys-Asp-Val-Tyr (thymopentin) representing amino acids 32−36 of the thymopoetin structure retained the biological activity of the larger peptide. Investigation by DiPerri *et al.*[4] elaborated the immunokinetics of thymopentin *in vivo*.

A series of clinical studies has suggested that thymopentin has value in the treatment of several immune-related disorders such as atopic dermatitis,[5] hemolytic anemia accompanying chronic lymphocytic leukemia,[6] Sezary's syndrome,[7] and decreased immune competency in elderly surgical patients.[8] During the course of clinical research, it was also found that the drug was effective in the treatment of patients suffering from severe rheumatoid arthritis.[9,10] Both subjective and objective measurements of patient status showed distinct improvement. Even though the drug was free of overt toxicity, the short half-life of approximately 30−60 s in plasma required applications two or three times weekly by a continuous iv infusion procedure.[11]

Thymopentin has also been investigated clinically for its effect on HIV illness.[12] Subjective improvement with weight gain and disappearance of fever was observed in early stage AIDS patients, but advanced cases complicated by opportunistic infections failed to respond adequately. More recently, thymopentin has been undergoing advanced clinical trials for treatment of AIDS patients.

Heavner and co-workers[13] conducted an initial structure−activity investigation of thymopentin by making variations in the peptide sequence. Twenty-nine analogs were synthesized and assessed *in vitro* for their ability to induce elevation of cyclic GMP levels in human T-cells and for competitive binding for the T-cell receptor. L-Arginine at position 1 and L-aspartic acid at position 3 were found to be indispensable for activity. It was interesting that substitution of L-glutamic for aspartic acid caused a loss of binding for the receptor. This peptide, splenin, isolated from bovine spleen, has shown a similar ability to induce T- and B-cell precursors.[14] The lysine at position 2 could be replaced by proline, but with some loss of activity when D-lysine or α-aminoisobutyrate was substituted. The valine at position 4 could be replaced by its D-isomer, alanine, or sarcosine. The tyrosine at position 5 could be replaced by phenylalanine, valine, or D-tyrosine with varying degrees of activity. Histidine, tryptophan, or ring-substituted tyrosine (3-Cl, 3-NO₂) were also suitable replacements.

Some attempts were made[15] to develop analogs with a longer half-life in plasma. N-Terminal acetylation or C-terminal amidation of the various analogs generally resulted in an enhancement of the half-life as compared to thymopentin. However, only the 2-proline analog retained activity following acetylation or amide formation at the C-terminus. Curiously, thymopentin itself retained activity following a combination of acetylation and C-terminal amidation.

Sisto *et al.*[16] reported a "retro-inverso" analog of thymopentin wherein the Arg-Lys CONH bond was
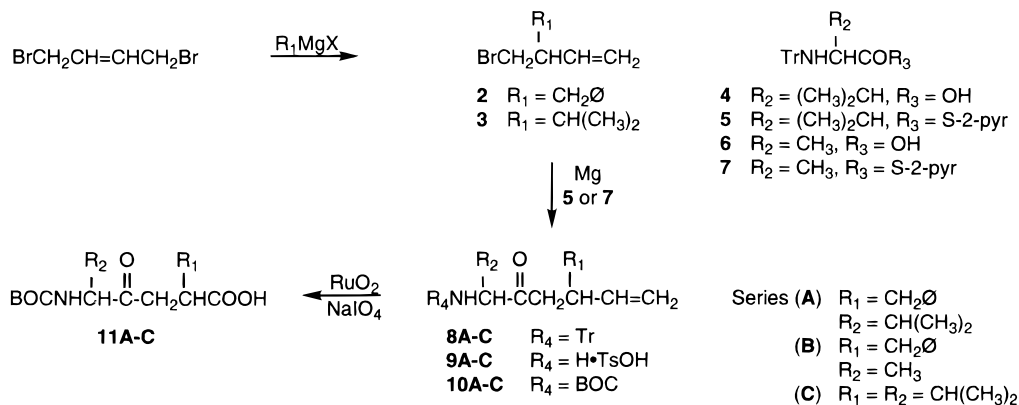
† SRI International.
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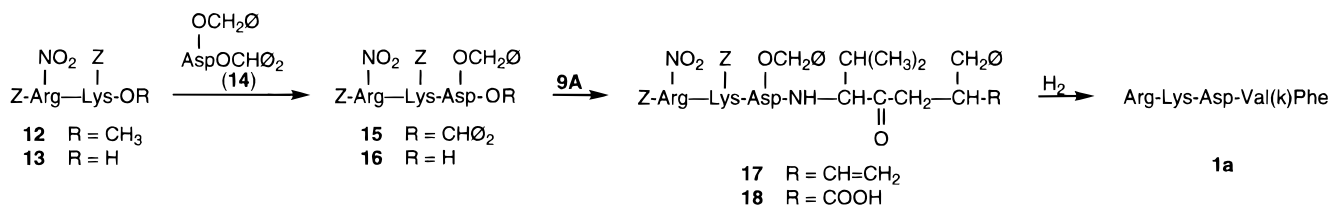§ Southern Research Institute.
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⊗ Abstract published in *Advance ACS Abstracts,* June 15, 1997.

Scheme 1

$$\text{BrCH}_2\text{CH=CHCH}_2\text{Br} \xrightarrow{\text{R}_1\text{MgX}} \underset{\overset{|}{\text{R}_1}}{\text{BrCH}_2\text{CHCH=CH}_2} \qquad \underset{\overset{|}{\text{R}_2}}{\text{TrNHCHCOR}_3}$$

2 $R_1 = CH_2\varnothing$
3 $R_1 = CH(CH_3)_2$

4 $R_2 = (CH_3)_2CH$, $R_3 = OH$
5 $R_2 = (CH_3)_2CH$, $R_3 = S\text{-}2\text{-pyr}$
6 $R_2 = CH_3$, $R_3 = OH$
7 $R_2 = CH_3$, $R_3 = S\text{-}2\text{-pyr}$

$$\downarrow \text{Mg} \quad \textbf{5 or 7}$$

$$\underset{\textbf{11A-C}}{\text{BOCNHCH-C-CH}_2\text{CHCOOH}} \xleftarrow[\text{NaIO}_4]{\text{RuO}_2} \underset{\substack{\textbf{8A-C} \ \ R_4 = Tr \\ \textbf{9A-C} \ \ R_4 = H\cdot TsOH \\ \textbf{10A-C} \ \ R_4 = BOC}}{\text{R}_4\text{NHCH-CCH}_2\text{CH-CH=CH}_2}$$

Series **(A)** $R_1 = CH_2\varnothing$
$\qquad\qquad R_2 = CH(CH_3)_2$
(B) $R_1 = CH_2\varnothing$
$\qquad R_2 = CH_3$
(C) $R_1 = R_2 = CH(CH_3)_2$

Scheme 2

$$\underset{\substack{\textbf{12} \ \ R = CH_3 \\ \textbf{13} \ \ R = H}}{\text{Z-Arg—Lys-OR}} \xrightarrow[\textbf{(14)}]{\text{AspOCH}\varnothing_2} \underset{\substack{\textbf{15} \ \ R = CH\varnothing_2 \\ \textbf{16} \ \ R = H}}{\text{Z-Arg—Lys-Asp-OR}} \xrightarrow{\textbf{9A}} \underset{\substack{\textbf{17} \ \ R = CH=CH_2 \\ \textbf{18} \ \ R = COOH}}{\text{Z-Arg—Lys-Asp-NH—CH-C-CH}_2\text{—CH-R}} \xrightarrow{\text{H}_2} \underset{\textbf{1a}}{\text{Arg-Lys-Asp-Val(k)Phe}}$$

reversed to NHCO. This unique analog was exceptionally stable to hydrolytic cleavage, but retention of activity was difficult to assess since the assay employed was not comparable with those of Heavner *et al.*[13,15]

This nontoxic immunomodulatory drug has demonstrated considerable potential for the treatment of primary immunodeficiency, autoimmune disorders (rheumatoid arthritis), and infectious disease. The primary drawback to the use of thymopentin in a chronic therapeutic mode is the very short half-life in plasma. Our strategy to improve stability with retention of activity featured systematic replacement of key amide bonds between amino acids 1−2, 3−4, or 4−5 with essentially isosteric, hydrolytically stable moieties such as ketomethylene, hydroxymethylene, or methylene amino groups. This methodology originally developed in our laboratories[17] and by the Szelke group[18] in the late 1970s is now frequently employed to obtain stable small peptide molecules. In this first paper of the series, we describe the synthesis and *in vitro* evaluation of analogs with stabilized bonds between amino acids 4−5 of thymopentin as represented by compounds of formula **1a−n**.
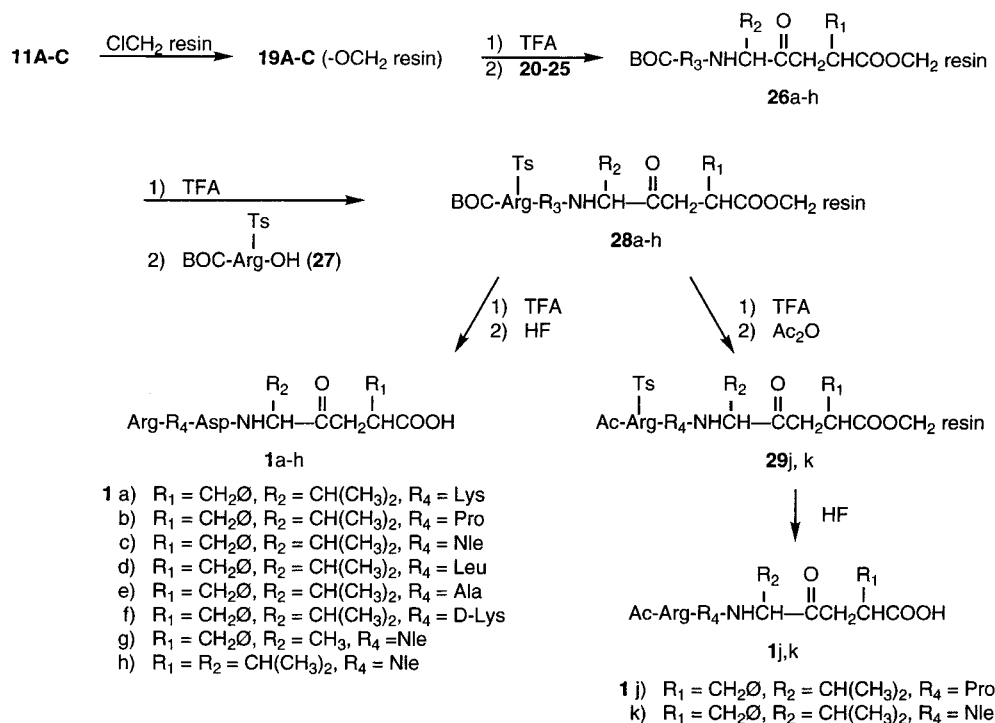
Chemistry

The structure−activity investigation was conducted primarily with a ketomethylene bond inserted between positions 4 and 5 and with phenylalanine in place of tyrosine [Val(k)Phe] with subsequent substitution of various amino acid units in place of L-lysine at position 2 of thymopentin. The substitution of Phe for Tyr at position 5 was made to reduce synthetic complexity in view of the observation that the phe group was an acceptable replacement in the straight peptide chain. Additional later variations at 4−5 were Ala(k)Phe- and Val(k)Val-containing analogs. In Scheme 1, the general route for synthesis of the three pseudodipeptide synthons is presented. In the Val(k)Phe unit (**11A**) the process involved reaction of benzyl Grignard reagent with 1,4-dibromo-2-butene to afford 3-(bromomethyl)-

4-phenyl-1-butene (**2**) in about 70% yield, accompanied by a major byproduct, diphenylethane. L-Valine was converted to its *N*-trityl derivative (**4**) by treatment with trimethylsilyl chloride followed by trityl chloride−Et₃N. The *N*-tritylvaline was then coupled with 2-mercapto-pyridine−dicyclohexylcarbodiimide to afford the crystalline *N*-tritylvaline−mercaptopyridine ester (**5**) in 20% yield from valine. The thiopyridyl ester was then allowed to react with an equivalent of the Grignard reagent prepared from **2** to yield the *N*-Tr keto olefin (**8A**) in 63% yield, as a mixture of diastereomers. The isomers were readily separated by flash chromatography on silica gel with 0.5% EtOAc in hexane to give isomer A (33% yield) and B (30%). When either isomer was treated with 1.1 equiv of *p*-toluenesulfonic acid in acetonitrile, the trityl was removed to afford the respective chiral amine salt (**9A**). The olefinic amino ketone salt was converted to its *N*-BOC derivative (**10A**) by treatment with di-*tert*-butyl dicarbonate and the olefin then oxidized to the pseudodipeptide acid (**11A**) with RuO₂−NaIO₄.[19] Preparation of the Ala(k)Phe synthon (**11B**) was conducted in a similar manner by reaction of the Grignard reagent from **2** with *N*-tritylalanine thiopyridine ester (**7**), followed by removal of the *N*-trityl group, blocking as *N*-BOC (**10B**) and oxidation as above. The Val(k)Val subunit (**11C**) required initial reaction of isopropylmagnesium bromide with 1,4-dibromobutene to give the bromo olefin (**3**) in 63% yield. Reaction of the Grignard reagent from **3** with *N*-tritylvaline thiopyridine ester (**6**) afforded the *N*-trityl keto olefin (**8C**) in 38% yield. Acidic removal of *N*-trityl yielded the amino ketone salt (**9C**), which was followed by conversion to **10C** and **11C** in the manner described above.
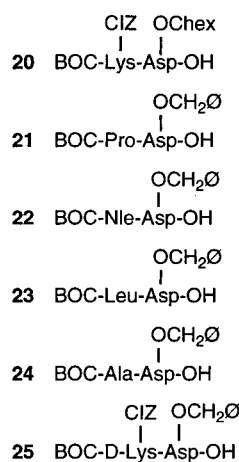
The initial approach to synthesis of a primary target, Arg-Lys-Asp-Val(k)Phe (**1a**) was by a solution phase process as shown in Scheme 2. ϵ-(Carbobenzyloxy)-L-lysine methyl ester was coupled with α-Cbz-nitroarginine to afford the di-Cbz-NO₂ ester (**12**),[20] which was carefully saponified to yield the blocked carboxylic acid

Scheme 3

$$11\text{A-C} \xrightarrow{\text{ClCH}_2 \text{ resin}} 19\text{A-C} \ (\text{-OCH}_2 \text{ resin}) \xrightarrow[\text{2) } 20\text{-}25]{\text{1) TFA}} \text{BOC-R}_3\text{-NHCH-CCH}_2\text{CHCOOCH}_2 \text{ resin}$$

BOC-R₃-NHCH-CCH₂CHCOOCH₂ resin with R₂, O, R₁

26a-h

$$\xrightarrow[\text{2) BOC-Arg-OH (27)}]{\text{1) TFA}}$$
(Ts)

BOC-Arg-R₃-NHCH—CCH₂-CHCOOCH₂ resin (Ts, R₂, O, R₁)

28a-h

1) TFA
2) HF

1) TFA
2) Ac₂O

Arg-R₄-Asp-NHCH—CCH₂CHCOOH (R₂, O, R₁)

1a-h

1 a) R₁ = CH₂Ø, R₂ = CH(CH₃)₂, R₄ = Lys
 b) R₁ = CH₂Ø, R₂ = CH(CH₃)₂, R₄ = Pro
 c) R₁ = CH₂Ø, R₂ = CH(CH₃)₂, R₄ = Nle
 d) R₁ = CH₂Ø, R₂ = CH(CH₃)₂, R₄ = Leu
 e) R₁ = CH₂Ø, R₂ = CH(CH₃)₂, R₄ = Ala
 f) R₁ = CH₂Ø, R₂ = CH(CH₃)₂, R₄ = D-Lys
 g) R₁ = CH₂Ø, R₂ = CH₃, R₄ = Nle
 h) R₁ = R₂ = CH(CH₃)₂, R₄ = Nle

Ac-Arg-R₄-NHCH—CCH₂CHCOOCH₂ resin (Ts, R₂, O, R₁)

29j, k

HF

Ac-Arg-R₄-NHCH—CCH₂CHCOOH (R₂, O, R₁)

1j, k

1 j) R₁ = CH₂Ø, R₂ = CH(CH₃)₂, R₄ = Pro
 k) R₁ = CH₂Ø, R₂ = CH(CH₃)₂, R₄ = Nle

Note: **26** and **28**
a   R₃ from **20**
b   R₃ from **21**
c, g, h   R₃ from **22**
d   R₃ from **23**
e   R₃ from **24**
f   R₃ from **25**

(CIZ) (OChex)
20 BOC-Lys-Asp-OH

(OCH₂Ø)
21 BOC-Pro-Asp-OH

(OCH₂Ø)
22 BOC-Nle-Asp-OH

(OCH₂Ø)
23 BOC-Leu-Asp-OH

(OCH₂Ø)
24 BOC-Ala-Asp-OH

(CIZ) (OCH₂Ø)
25 BOC-D-Lys-Asp-OH

(**13**). The blocked dipeptide was then coupled with L-aspartic acid blocked as the β-benzyl-α-benzhydryl ester (**14**) to give the tripeptide ester (**15**). Selective cleavage of the benzhydryl group by trifluoroacetic acid (TFA)/anisole afforded the blocked tripeptide (**16**) in 59% overall yield from compound **13**.[13] Coupling of **16** with **9A** produced the blocked olefin (**17**) in 88% yield. Oxidation of **17** with RuO₂−NaIO₄ readily gave the acid (**18**), which was deblocked by hydrogenolysis over a Pd catalyst to ultimately give **1a** as A- and B-isomers. This solution phase process was not employed further for synthesis of other target compounds; however, the pseudopentopeptide **1a** was found to be identical by HPLC to material prepared by solid-phase methodology described below.

In Scheme 3, a modified process making use of Merrifield resin technology is outlined. Each of the blocked pseudodipeptide acids (**11A−C**) were attached to Merrifield chloromethyl resin[21] to give the *N*-BOC-esterified resin preparations (**19A−C**). Cleavage of the *N*-BOC group with trifluoroacetic acid followed by coupling of an appropriate *N*-BOC dipeptide unit containing L-aspartic acid blocked at the β-carboxyl as a benzyl or cyclohexyl ester (**20−25**) gave the blocked pseudotetrapeptides bound to resin (**26a−h**). Previous work in our laboratory[26] had shown that BOC-protected amino acids successfully coupled with ketomethylene-containing pseudodipeptides to give the desired pseudo-tripeptides. However, BOC removal resulted in the formation of cyclic Schiff's bases between the ketone and liberated N-terminus. These cyclic imines would not take part in subsequent coupling reactions, thus frustrating the usual chain-building process whereby N-blocked single amino acids are sequentially coupled at the N-terminus. We found that coupling protected dipeptides to ketomethylene-containing pseudodipeptides does not give rise to truncated products. Removal of the *N*-BOC by treatment of **26** with TFA followed by coupling with α-*N*-BOC-γ-*N*-tosylarginine (**27**) activated as the benzotriazole ester afforded the resin-bound, blocked pentapeptides (**28a−h**). The final deblocking and removal from resin was accomplished by digestion of **28** with TFA followed by hydrogen fluoride to yield the target pseudopentapeptides (**1a−h**). The *N*-acetyl analogs **1j** and **1k** were prepared by removal of the *N*-BOC from **28** with subsequent acetylation by acetic

Scheme 4

BOC-NMe-Nle   ⟶   H
               NMeNle-O-*t*-Bu
               30

           OChex
           |
           Asp-O-*t*-Bu
                           OChex
                           |

Z_3Arg ⟶ Z_3Arg-NMeNle-OR   ⟶(33)⟶ Z_3Arg-NMeNle-Asp-OR

          31   R = *t*-Bu   **34**   R = *t*-Bu
          32   R = H   **35**   R = H

                         OChex
                         |
19A or 19C ⟶ Z_3Arg-NMeNle-Asp-Val(k)Phe-O-CH$_2$ resin **36**

                         OChex
                         |
             Z_3Arg-NMeNle-Asp-Val(k)Val-O-CH$_2$ resin **37**

                   ↓ HF

         1l   Arg-NMeNle-Asp-Val(k)Phe
         1m   Arg-NMeNle-Asp-Val(k)Val
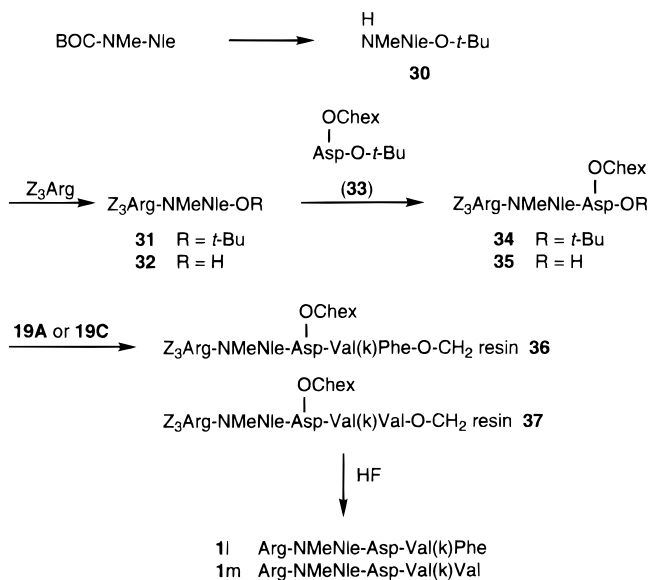
anhydride (**29j,k**) prior to complete deprotection with hydrogen fluoride.

The Arg-NMeNle-Asp-Val(k)Phe analog (**1l**) and the related Val(k)Val analog (**1m**) were synthesized by the method shown in Scheme 4. Tripeptide intermediate **34** was synthesized unconventionally from the N-terminal amino acid to the C-terminal amino acid. Our attempts to follow the conventional approach, i.e., coupling Z_3ArgOH with NMeNle-Asp(OCHx)OtBu, gave little or no product. Thus *N*-Me-*N*-BOC-norleucine was allowed to react with isobutylene in dioxane containing H_2SO_4 to give the *tert*-butyl ester of *N*-methylnorleucine (**30**) with concomitant loss of the *N*-BOC group. Coupling of **30** with tri-CBZ-arginine afforded the Z_3Arg-NMeNle-*t*-Bu ester (**31**), which was subsequently treated with TFA to cause selective cleavage of the *t*-Bu ester to give the acid (**32**). Coupling of **32** with aspartic acid α-*t*-Bu-β-cyclohexyl diester (**33**) yielded the blocked tripeptide (**34**). The *t*-Bu group was again cleaved by TFA, and the resulting acid (**35**) was coupled with either **19A** or **19C** to produce the resin-bound blocked pentapeptide **36** or **37**, respectively. Cleavage from the resin and blocking groups by treatment with HF gave **1l** and **1m**.

As explained above it was necessary to use less conventional coupling strategies involving activation of blocked dipeptides with pseudodipeptides. Such procedures are occasionally employed in peptide construction, but there is heightened concern for racemization. Either HOBt/DCC or HOSu/DCC activation strategies[27] were used to minimize racemizations that can occur when coupling peptide fragments. It should be noted that evidence of racemic products was not observed in HPLC traces nor NMR spectra except in instances where racemic intermediates were deliberately employed. In those latter cases the racemic diastereomers were readily detected and separated by HPLC.

The hydroxyethano analog (**1n**) was prepared from the ketomethylene peptide (**1c**) by reduction with LiBH$_4$ as shown in Scheme 5. The reduction product was characterized as the lactone (**38**) reflecting a mixture of stereoisomers. The lactone could be opened to the hydroxy acid (**1n**) by treatment with mild alkali. The pseudopeptide was retained in the form of its sodium

salt for purposes of biological evaluation since acidification caused reversal to the lactone form.

Biological Evaluation

Receptor Binding. *In vitro* studies characterized the thymopentin (TP-5) analogs by receptor binding using the [^{3}H]TP-5 competition assay with CEM cells and isolated thymocytes. A ranking of the various analogs was developed based on statistical analysis of the competitive nature of each of the analogs tested in the [^{3}H]TP-5 binding assays. The analogs were ranked with respect to overall competitive activity relative to authentic TP-5. The competition assay relied on the ability of nonradiolabeled TP-5 or nonlabeled analog to compete for the binding sites of [^{3}H]TP-5. TP-5 binding assays were carried out using a tritiated sample having a specific activity of 37 Ci/mmol. In the absence of a competitor, TP-5 binding was a function of time of exposure and temperature. The mean total counts of radiolabeled TP-5 bound in the absence of competition by nonradiolabeled TP-5 was 3078 cpm ($N = 47$). Competition by nonradiolabeled TP-5 or TP-5 analogs decreased the amount of radioactive TP-5 bound in proportion to concentration and relative affinity for the thymopoietin receptor. In a representative experiment the counts decreased from 3175 cpm at 10^{-5} M TP-5 to 1879 cpm at 10^{-4} M and to 1133 at 10^{-3} M. In comparison, the levels of radioactivity decreased from 2892 at 10^{-5} M for a representative TP-5 analog to 2768 at 10^{-4} M and 1944 at 10^{-3} M. Each experiment was repeated at least twice with both CEM cells and isolated thymocytes. Competition with nonradiolabeled TP-5 was always carried as a positive control for comparison and typically resulted in 1150 cpm ($N = 75$) at 10^{-3} M.

Parameters of the binding assay which have been examined include the temperature, time dependency, and relationship of radiolabeled TP-5 to total binding. A comparison of binding at 4, 25, and 37 °C showed maximal binding occurred at 37 °C over a period of 30 min (Table 1). Longer times did not further increase binding of TP-5.

To validate the TP-5 binding assay, competition studies were carried out in the presence of 0.1% sodium azide to inhibit cell metabolism and block internalization of the peptide. Under these conditions, identical competition curves were obtained for inhibition of [^{3}H]-TP-5 binding by authentic TP-5 and TP-5 analogs as in the absence of sodium azide. These data show that reduction in [^{3}H]TP-5 binding following the addition of excess TP-5 or analogs represents decreased binding of the [^{3}H]TP-5 at the cell surface and is not a consequence of internalization of the radiolabeled peptide.

Each analog was analyzed in terms of the mean level of radioactivity remaining bound to CEM cells in the presence of increasing concentrations of nonlabeled competitor analog. The concentration of CEM cells was always constant. The data represent the means of replicate determinations at concentrations of added inhibitor at 10^{-4} and 10^{-3} M since these levels were typically the range in which the greatest amount of competition was observed. Under the binding conditions used, the radiolabeled TP-5 was stable to degradation, resulting in the mean level of total counts of radiolabeled TP-5 being bound of 3078 ($N = 47$); as stated above, authentic unlabeled TP-5 reduced the

Scheme 5
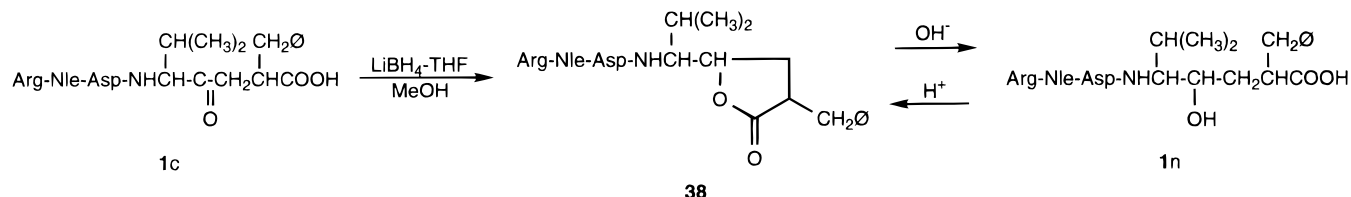
Table 1. Effect of Temperature on [³H]TP-5 Binding to CEM Cells[a]

temp (°C)	[³H]TP-5 bound (cpm)	
	0 min	30 min
4	341	2821
25	432	4566
37	579	5522

[a] CEM cells were obtained from the American Type Culture Collection (Rockville, MD). The cells were resuspended at a final concentration of 5×10^6 cells/0.1 mL of binding buffer. Radiolabeled TP-5 (548 000 cpm) was added, and the cells were harvested immediately or incubated for 30 min at 4, 25, or 37 °C. At the end of incubation, the cells were diluted with 1 mL of ice-cold binding buffer and washed two times by centrifugation. Radioactivity was determined by scintillation counting.

Table 2. TP-5 Analog Binding to CEM Cells[a]

compound tested	no. of trials	binding competition mean ± SEM	efficacy
none	47	3078 ± 49	
1a-A, Arg-Lys-Asp-Val(k)Phe	8	1735 ± 207	70
1a-B, Arg-Lys-Asp-Val(k)Phe	8	1673 ± 209	73
1b-A, Arg-Pro-Asp-Val(k)Phe	6	1843 ± 130	64
1b-B, Arg-Pro-Asp-Val(k)Phe	6	2000 ± 111	56
1c-A, Arg-Nle-Asp-Val(k)Phe	12	1254 ± 96	95
1c-B, Arg-Nle-Asp-Val(k)Phe	12	1453 ± 130	84
1d-A, Arg-Leu-Asp-Val(k)Phe	6	1851 ± 437	65
1e-A, Arg-Ala-Asp-Val(k)Phe	6	1780 ± 315	67
1f-A, Arg-D-Lys-Asp-Val(k)Phe	6	2044 + 307	54
1g-A, Arg-Nle-Asp-Ala(k)Phe	6	1677 ± 243	73
1g-B, Arg-Nle-Asp-Ala(k)Phe	6	1715 ± 392	71
1h-A, Arg-Nle-Asp-Val(k)Val	6	1600 ± 150	77
1h-B, Arg-Nle-Asp-Val(k)Val	6	1713 ± 150	71
1j-A, Ac-Arg-Pro-Asp-Val(k)Phe	6	2252 ± 88	43
1k-A, Ac-Arg-Nle-Asp-Val(k)Phe	6	2216 ± 148	45
1l-A, Arg-NMeNle-Asp-Val(k)Phe	6	2356 ± 235	37
1m-A, Arg-NMeNle-Asp-Val(k)Val	6	2356 ± 200	37
1n-A, Arg-Nle-Asp-Val(CHOH)Phe	6	2400 ± 232	35
1n-A lactone	6	2771 ± 97	16
TP-5, Arg-Lys-Asp-Val-Tyr	75	1150 ± 60	100

[a] The competitive nature of the analogs in the isolated thymocyte binding assay was comparable to the CEM cell binding assay. CEM cells were resuspended at a final concentration of 5×10^6 cells/0.1 mL of binding buffer. For competition assays, dilutions of TP-5 or TP-5 analogs were added to the cells over a concentration range from 10^{-6} to 10^{-3} M. Radiolabeled TP-5 (548 000 cpm) was added, and the cells were incubated for 30 min and at 25 °C. At the end of incubation, the cells were diluted with 1 mL of ice-cold binding buffer and washed two times by centrifugation. Radioactivity was determined by scintillation counting.

binding to 1150 cpm ($N = 75$). The determination of the efficacy for each compound was based on the fact that the reduction in binding was statistically significant from that of authentic TP-5 using analysis of variance and Student's two-sample t-test (two-tailed). Efficacy of the analog represents a comparison with the competitive nature of authentic TP-5; counts remaining after competition were subtracted from total binding of TP-5 and divided by the counts reduced by authentic TP-5 (Table 2).

The results presented in Table 2 show that substitution of a ketomethylene bond for the normal amide bond

between amino acids 4 and 5 did not destroy binding to CEM cells. However, on the average, binding was reduced about 20−30% as compared with TP-5. The previous work of Heavner et al.[13] indicated that arginine and aspartic acid were essential at positions 1 and 3, respectively. In this investigation encompassing the substitution of a stabilized bond between 4 and 5 we have varied the amino acids at positions 2, 4, and 5. As mentioned earlier it was necessary to establish whether phenylalanine could be substituted for tyrosine at the carboxy terminus in order to minimize synthetic complexity. This was indeed a valid modification since TP-5 and TP-5 (5-Phe) gave very similar binding curves. When the ketomethylene bond was inserted as in **1a** [Val(k)Phe], an acceptable decrease in binding of only 30% was observed for the **1a-A** isomer and 27% for the **1a-B** isomer. (A and B isomer designations refer to the moiety −C(O)CH₂CH(R)CO and are arbitrarily assigned on the basis of chromatographic mobility of the initial ketomethylene intermediates encountered during the synthesis or upon first separation of peptide isomers. There is no established relevance to absolute configuration.)

Further structure−activity investigation was then conducted with maintenance of the Val(k)Phe moiety while varying the amino acid at position 2. Insertion of proline at 2 (**1b**) caused an additional decrease in binding for both isomers, while substitution with leucine (**1d-A**) had a similar effect. Replacement of the amino acid at 2 with alanine (**1e-A**) gave a result similar to that of **1a**. However, insertion of norleucine in place of the lysine moiety caused only a minimal decrease in binding (5% for **1c-A** and 16% for **1c-B**) at position 2. Since the norleucine substitution at 2 was minimally disruptive to binding, this change was combined with replacement of the Val(k)Phe moiety by Ala(k)Phe (**1g**). Both isomers of **1g** gave binding results similar to that of **1a**.

Other variations were made about the N-terminal region for purposes of decreasing enzymatic cleavage of the peptide bond between positions 1 and 2. Replacement of lysine at 2 with D-lysine (**1f-A**) caused a binding decrease of 46%. N-Terminal acetylation as in **1j-A** and **1k-A** was very detrimental to binding (57% and 55%, respectively). Insertion of N-methylnorleucine (**1l**) at the 2-position was significantly disruptive to binding (63% decrease), but conferred complete hydrolytic stability as discussed below.

It had also been noted[13] that valine could be substituted at position 5 of thymopentin with retention of activity. Insertion of a Val(k)Val grouping at 4−5 and norleucine at 2 as in **1h** was acceptable in terms of binding decrease (23% and 29% for isomers A and B, respectively). Stabilization of the 1−2 bond with N-Menorleucine (**1m-A**) again caused a major decrease in binding (63%).

Table 3. Stability of TP-5 and TP-5 Analogs in Mouse and Human Plasma

compd tested	half-life[a] (min)		
	mouse		human heparinized
	EDTA-treated	heparinized	
TP-5	3.1	0.8	1.5
1a-A	4.9	2.0	2.0
1b-A	17.0	4.5	6.0
1c-A	21.1	5.5	1.9
1g-A	20.0		-
1h-A		1.2	2.4
1h-B		2.6	2.4
1k-A	not degraded		5.1
1l-A		not degraded	not degraded
1m-A		not degraded	not degraded
1m-B		not degraded	not degraded

[a] Half-life of TP-5 and TP-5 analogs were determined by HPLC methods that permitted quantitative resolution of the decrease of TP-5 and the appearance of degradation products by absorbance. EDTA-treated and heparinized serum was collected to prevent removal of clotting components.

We also tested whether the ketomethylene bond at 4−5 could be replaced with hydroxyl. Reduction of the ketone in the tightly bound **1c-A** caused an unacceptable decrease in binding for **1n-A** in the open chain or lactone forms.

Stability Studies. *In vitro* studies also included an analysis of the stability of TP-5 and TP-5 analogs in mouse and human plasma. The plasma half-life of each target compound was determined following incubation in plasma for variable times. The analysis protocol was based on published studies for quantitation of TP-5 degradation products using HPLC techniques.[16] The analysis of the degradation of TP-5 can be visualized and quantified by the disappearance of the test compound simultaneous with the appearance of degradation products.

The results of analysis of plasma half-life have been completed for a series of analogs in mouse plasma that was prepared either by EDTA treatment or by heparin treatment of serum. The data for the half-life of selected compounds in mouse plasma is presented below in Table 3. Results for compounds tested in human heparinzed plasma are also presented in Table 3.

All of the compounds except **1h-A** (1.2 min) showed significant enhancement of their half-lives in mouse serum as compared with TP-5 (0.8 min). It is interesting that substitution of proline (**1b-A**) or norleucine (**1c-A**, **1g-A**) was particularly beneficial to prolongation of half-life. As mentioned earlier N-methylation of the 2-norleucine prevented degradation (**1l-A** and **1m-A,B**). It was more difficult to achieve significant stability increases over TP-5 in human serum. We found TP-5 to have a half-life of 1.5 min in human serum, while four compounds (**1a-A**, **1c-A**, **1h-A**, and **1h-B**) showed half-lives in the range 1.9−2.4 min. Compound **1b-A** (2-Pro) had a half-life of 6.0 min, reflecting the increased stability of the Arg-Pro bond. However, *N*-methylnorleucine at position 2 again conferred complete stability in human as well as mouse sera as seen for **1l-A** and **1m-A,B**. It is apparent that stabilization of the 4−5 bond is beneficial to stability in human serum, but interference with hydrolysis at the 1−2 bond is most important. These results tend to confirm those of Tischio et al.[22] who found the Arg−Lys bond to be the primary site of enzymatic degradation.

Discussion

The proposed mechanism of action of thymopoietin appears to depend on receptor-mediated binding of the hormone to target immune cells. Several investigations have demonstrated that a pentapeptide, thymopentin, derived from the whole molecule reproduces the effects of the parent hormone. The action of thymopoietin appears to involve the recruitment and maturation of T-lymphocytes, either as pre-thymocytes or during thymic maturation. As a result, biological effects of the fragment, thymopentin, have focused on immunological parameters of T-cell metabolism, including release of cyclic GMP and T-cell receptor binding. A number of studies have shown that the T-cell line, CEM cells, function well as a source of T-cell for analysis of thymopentin.

Our study used the criteria of CEM cell receptor binding as an index for comparison of new analogs with authentic thymopentin. The effectiveness of receptor binding provides a direct method for assessing structure/function relationships with new analogs through a competition assay. The addition of nonradiolabeled analog was shown to display the binding of radiolabeled TP-5 and thus provides a means for reproducible quantification of differences in the molecules. Under our conditions, the TP-5 binding assay was concentration dependent, temperature dependent, and time dependent.

The binding assays were corroborated with analysis of serum half-life studies which showed that the analogs developed exhibited extended serum stability. However, the efficacy in the binding assay was not immediately comparable to serum stability. Insertion of 4−5 ketomethylene did not severely reduce binding to CEM cells, but CHOH decreased binding by a factor of about 3. Incorporation of a proline residue at the 2-position as in **1b-A** gave an analog nearly equal to TP-5 in binding potency and with a significant increase in serum stability. When *N*-methylnorleucine was placed at the 2-position, complete stability was achieved, but with considerable loss of binding capability as in **1l-A** and **1m-A**. However, compounds **1l-A** and **1m-A** could still demonstate useful effects *in vivo* as a result of their long half-lives in plasma.

Experimental Section

Elemental analyses were obtained from Galbraith Laboratories, Knoxville, TN. Values were within 0.4% except as noted in parentheses following the element. ^{1}H- and ^{13}C-NMR spectra were determined with a Varian 400A or a JEOL FX90Q spectrometer. Mass spectra were obtained with a Ribermag R10-10C MS or a ZAB-2EQ VG reverse geometry high-resolution instrument in the case of final target peptides. Reverse phase HPLC analyses were conducted on a Waters Associates Novapak C18 column. The solvent systems were 10−30% CH_3CN/0.10% TFA.

Designation of isomers was made arbitrarily on the basis of chromatographic behavior at the first point of separation whether intermediate or final product. Isomer A was assigned to the faster moving component and B to the slower for thin layer or HPLC chromatograms.

Less common abbreviations used in the experimental description or test are as follows: ClZ (*p*-chlorobenzyloxycarbonyl), C-Hex (cyclohexyl), (BOC)$_2$O (di-*tert*-butyl dicarbonate), DIEA (diisopropylethylamine), MBHA (*p*-methylbenzhydrylamine resin), BOP (benzotriazol-1-yloxy)tris(dimethylamino)-phosphonium hexafluorophosphate).

3-(Bromomethyl)-4-phenyl-1-butene (2). Benzylmagnesium chloride (75 mL, 2 M) in THF (Aldrich, equivalent to 0.15 mol) and 2.86 g of CuI was stirred at 0 °C under argon for 15 min and then at room temperature for 25 min. The mixture was added to a −60 °C solution of 21.4 g (0.1 mol) of 1,4-dibromo-2-butene in 150 mL of anhydrous Et$_2$O. The mixture was stirred at −60 °C for 30 min and then at room temperature for 16 h. The mixture was poured into 400 mL of 50% saturated NH$_4$Cl/ice and extracted with Et$_2$O. The organic layer was dried (Na$_2$SO$_4$) and concentrated to a yellow oil, 21.2 g. NMR shows 30% dibenzyl present, along with the product estimated at 70%: NMR (CDCl$_3$) δ 2.86 (m, 3H, CH$_2$-Ar + C*H*CH=CH$_2$), 2.86 (s, CH$_2$ of dibenzyl), 3.23 (d, 2H, CH$_2$-Br), 4.94 (m, 2H, C*H$_2$*=CH), 5.65 (m, 1H, C*H*=CH$_2$), 7.2 (m, Ar).

3-(Bromomethyl)-4-methyl-1-pentene (3). A mixture of 60 mL of 2 M isopropylmagnesium chloride (0.12 mol) and 232 mg (1.45 mmol) of CuI was stirred under argon for 1 h. The reagent was transferred over 15 min via a cannula to a stirred solution of 21.2 g (0.1 mol) of 1,4-dibromo-2-butene in 200 mL of Et$_2$O at −78 °C. The resulting mixture was stirred at ambient temperature for 4 h and added to 300 mL of saturated NH$_4$Cl. The Et$_2$O layer was separated, washed with 200 mL of brine, dried over MgSO$_4$, and evaporated *in vacuo* to afford 11.2 g (63%): bp 60 °C/15 mmHg; NMR (CDCl$_3$) δ 0.83 (t, 6H, CH$_3$), 1.80 (m, 1H, C*H*Me$_2$), 2.10 (m, 1H, C*H*CH=CH$_2$), 3.35 (d, 2H, CH$_2$Br), 5.0 (m, 2H, CH=C*H$_2$*), 5.50 (m, 1H, C*H*=CH$_2$).

***N*-Trityl-L-valine 2-Mercaptopyridine Ester (5).** L-Valine (25.0 g, 0.21 mol) and 27.1 mL (23.2 g, 0.21 mol) of chlorotrimethylsilane in 500 mL of CHCl$_3$/CH$_3$CN (5/1) was stirred at reflux under argon for 2 h. The mixture was then cooled to 0 °C and treated, dropwise, with 59.3 mL (43.0 g, 0.43 mol) of triethylamine, then dropwise, with a solution of 59.4 g (0.21 mol) of triphenylmethyl chloride in 200 mL of CHCl$_3$. The mixture was stirred at room temperature for 1.5 h and then partitioned between 750 mL of 5% citric acid and 750 mL of Et$_2$O. The organic layer was washed with 1 M NaOH (2 × 500 mL) and then water (500 mL). The aqueous washes were combined, cooled to 0 °C, and neutralized (pH = 6−7) with glacial HOAc. The mixture was extracted with Et$_2$O (3 × 500 mL). The organic extracts were combined, dried over Na$_2$SO$_4$, and concentrated. The white foam [27.3 g of *N*-Tr-valine (4)] was dissolved in 300 mL of EtOAc and treated with 8.5 g (76.4 mmol) of 2-mercaptopyridine. The solution was cooled to 0 °C and treated with 15.75 g (76.4 mmol) of dicyclohexylcarbodiimide. The solution was stirred at 0 °C for 3 h and then at room temperature for 16 h. The mixture was filtered and the filtrate concentrated. Crystallization of the residue from hexane/EtOAc (10/1) yielded **5** as a white crystalline product, 18.4 g (20% yield from valine). Anal. Calcd for C$_{29}$H$_{28}$N$_2$OS·¼H$_2$O: C, H, N.

***N*-Trityl-L-alanine (6).** L-Alanine (40.0 g, 0.45 mol) and chlorotrimethylsilane (48.8 g, 0.45 mol) in a mixture of CHCl$_3$ (640 mL) and CH$_3$CN (160 mL) were stirred at reflux under argon for 2 h. The mixture was cooled to 0 °C and treated dropwise with triethylamine (90.9 g, 0.90 mol) and then dropwise with a solution of triphenylmethyl chloride (125.45 g, 0.45 mol) in CHCl$_3$ (400 mL). The mixture was stirred at room temperature for 1 h and then quenched by adding MeOH (450 mL). After evaporation to a foam, the residue was partitioned between ice-cold 5% citric acid (1000 mL) and Et$_2$O (1500 mL), and the organic layer was extracted with 1 N NaOH (2 × 1000 mL). The combined aqueous layers were neutralized with HOAc and extracted with EtOAc (3 × 500 mL). The organic extracts were combined, dried over MgSO$_4$, and evaporated to give 133.8 g (90%); NMR (CDCl$_3$) δ 1.21 (d, 3H, CH$_3$), 3.32 (m, 1H, CH), 6.45 (bs, 1H, NH), 7.00−7.36 (m, 15H, Ar).

***N*-Trityl-L-alanine 2-Mercaptopyridine Ester (7).** DCC (9.1 g, 44.1 mmol) was added to a solution of **6** (14.2 g, 42.8 mmol) and 2-mercaptopyridine (4.75 g, 42.8 mmol) in EtOAc (160 mL), and the resulting mixture was stirred for 4 days at room temperature. The precipitate was removed by filtration and the filtrate evaporated to give a light yellow solid. The material was crystallized from EtOAc−hexane to give 4.18 g

(23%): NMR (CDCl$_3$) δ 0.98 (d, 3H, CH$_3$), 3.56 (m, 1H, CH), 7.00−7.60 (m, 18H, Ar, pyr), 8.40 (m, 1H, pyr-3-H).

6-(Tritylamino)-7-methyl-3-benzyl-1-octen-5-one (8a). Magnesium (4.4 g) in 150 mL of dry THF was treated with 0.1 mL of ethylene dibromide and a catalytic amount of I$_2$. A solution (10 mL) of 3-(bromomethyl)-4-phenyl-1-butene (**2**) (28 g of 70% pure, equivalent to 19.6 g, 87 mmol) in 70 mL of dry THF was added, followed by heating under argon to 50−60 °C. After the reaction started, the remaining bromide was added while maintaining a 50−60 °C reaction temperature. The mixture was stirred at gentle reflux for 2 h and then cooled to room temperature, followed by addition of *N*-trityl-L-valine mercaptopyridine ester (**5**, 28 g, 62 mmol) all at once. The mixture was stirred at 50−60 °C for 2 h, cooled to 0 °C, poured into saturated NH$_4$Cl, and extracted with Et$_2$O. The organic layer was dried (MgSO$_4$) and concentrated. Flash chromatography on silica gel (0.5% EtOAc in hexane) allowed separation of diastereomers. The total yield for both isomers was 19.1 g (63%).

Isomer A (10.0 g): MS *m/e* 486 (M + H); TLC (0.5% EtOAc in hexane); R_f 0.50; NMR (CDCl$_3$) δ 0.82 (d, 3H, CH$_3$), 1.03 (d, 3H, CH$_3$), 2.08 (m, 2H, CHMe$_2$, CH-vinyl), 2.10−2.65 (m, 4H, CH$_2$CO, ArCH$_2$), 3.16 (m, 1H, NH), 3.26 (m, 1H, CHNH), 4.70 (dd, 1H, CH$_2$=C), 4.84 (dd, 1H, CH$_2$=C), 5.42 (m, 1H, CH=C), 6.97−7.56 (m, 20H, Ar). Anal. Calcd for C$_{35}$H$_{37}$NO: C, H, N.

Isomer B (9.1 g): TLC (0.5% EtOAc in hexane); R_f 0.40; NMR (CDCl$_3$) δ 0.84 (d, 3H), 1.10 (d, 3H), 2.00 (m, 2H), 2.20−2.65 (m, 4H), 3.05 (m, 1H), 3.30 (m, 1H), 4.76 (t, 1H), 4.90 (dd, 1H), 5.42 (m, 1H), 6.97−7.54 (m, 20H).

6-(Tritylamino)-3-benzyl-1-hepten-5-one (8b). The Grignard reagent was prepared as described above using bromide **2** (2.2 g, 9.86 mmol) and Mg turnings (500 mg) in THF (50 mL). A solution of **7** (4.18 g, 9.86 mmol) in THF (50 mL) was added dropwise over 15 min. The resulting mixture was stirred at 65 °C for 2 h and then at room temperature overnight. The solution was partitioned between Et$_2$O (400 mL) and saturated NH$_4$Cl (400 mL). The organic layer was washed with brine, dried over MgSO$_4$, and evaporated to give a pale yellow syrup. The material was purified by flash chromatography, eluting with 2.5% EtOAc in hexane to give 1.98 g (44%) as a colorless syrup: NMR (CDCl$_3$) δ 1.15 (m, 3H, CH$_3$), 2.00 (m, 1H, CH vinyl), 2.22 (m, 1H, CHCO), 2.46 (m, 2H, ArCH$_2$), 3.21 (m, 2H, CHCO, CHN), 4.60−4.88 (m, 2H, CH$_2$=C), 5.28−5.72 (m, 1H, CH=C), 6.95−7.52 (m, 20H, Ar).

7-Methyl-6-(tritylamino)-3-isopropyl-1-octen-5-one (8c). To Mg turnings (1.10 g, 45.6 mmol) in Et$_2$O (20 mL) was added 1,2-dibromoethane (2.70 g, 14.4 mmol) at a rate that maintained reflux. The resulting mixture was refluxed for an additional hour, followed by the dropwise addition of 3-(bromomethyl)-4-methyl-1-pentene (**3**) (2.50 g, 14.1 mmol) in Et$_2$O (10 mL) over a 30-min period. Reflux was continued for 1 h; then the mixture was cooled to room temperature. The resulting Grignard solution was transferred via cannula to a cooled (0 °C) solution of **5** (3.18 g, 7.5 mmol) in THF (30 mL). The reaction was allowed to proceed at room temperature overnight. The material was partitioned between Et$_2$O (300 mL) and saturated NH$_4$Cl (300 mL). The organic layer was washed with brine (2 × 200 mL), dried over MgSO$_4$, and evaporated. The material was purified with filter-pad chromatography (30 mL bed volume of silica gel) using a step gradient with CCl$_4$ and MeCl$_2$ as the eluants (0%, 25%, 50%, 75%, 100% MeCl$_2$). Product fractions were combined and evaporated to give 1.26 g (38.3%) of product as a clear syrup: MS *m/e* 440 (M + H$^+$); NMR (CDCl$_3$) δ 0.65−1.05 (m, 9H, CH$_3$), 1.14 (dd, 3H, CH$_3$), 1.23−1.68 (m, 2H, 2 × CH), 1.80−2.45 (m, 3H, CH$_2$CO, C*H*(CH=CH$_2$)), 3.18 (dd, 1H, NH), 3.43 (dd, 1H, CHN), 4.77−5.12 (m, 2H, vinyl), 5.20−5.72 (m, 1H, CH vinyl), 7.18−7.65 (m, 15H, Ar); ^{13}C-NMR (CDCl$_3$) 210.3 and 211.08 (ketone isomers). Anal. Calcd for C$_{31}$H$_{37}$NO·0.2CHCl$_3$: C, H, N.

7-Methyl-6-amino-3-benzyl-1-octen-5-one *p*-Toluenesulfonate (9a). A mixture of the *N*-trityl ketone **8a** (1.06 g, 2.17 mmol), 0.46 g (2.39 mmol) of *p*-toluenesulfonic acid, and 50 mL of CH$_3$CN was stirred at room temperature for 1 h. The solvent was removed *in vacuo* to leave a white solid residue which was triturated with 50 mL of hexane−Et$_2$O (3:

2). The white solid was collected, washed with the same solvent, and dried to leave 0.80 g (88%) of the tosylate salt: NMR (CDCl$_3$) δ 0.82 (q, 6H, CH$_3$), 2.43 (m, 9H, ArCH$_3$, ArCH$_2$-CH, CH$_2$C=O, C*H*Me$_2$), 3.94 (m, 1H, C*H*NH$_2$), 4.64 (m, 2H, vinyl), 5.44 (m, 1H, vinyl), 6.90 (m, 5H, C$_6$H$_5$), 7.66 (m, 4H, C$_6$H$_4$).

7-Methyl-6-*N*-(*tert*-butyloxycarbonyl)-3-benzyl-1-octen-5-one (10a). A solution of 0.80 g (1.9 mmol) of the tosylate salt (**9a**) and 0.84 g (3.8 mmol) of di-*tert*-butyl dicarbonate in 50 mL of dichloromethane was treated, dropwise over 10 min, with 0.21 g (2.1 mmol) of triethylamine in 2 mL of MeCl$_2$. The solution was stirred at room temperature for 16 h and washed successively with 0.5 N HCl, H$_2$O, 1 M Na$_2$CO$_3$, and H$_2$O. The organic layer was dried (Na$_2$SO$_4$) and concentrated. Flash chromatography of the residue on silica gel (50% hexane/MeCl$_2$) yielded 0.40 g of a colorless syrup (61%): NMR (CDCl$_3$) δ 0.80 (q, 6H, CH$_3$), 1.36 (s, 9H, *t*-Bu), 2.00 (m, 1H, C*H*Me$_2$), 2.53 (m, 4H, ArCH$_2$, CH$_2$CO), 2.86 (m, 1H, C*H*CH=CH$_2$), 4.10 (m, 1H, C*H*NH), 4.93 (m, 3H, vinyl, NH), 5.56 (m, 1H, vinyl), 7.04 (m, 5H, C$_6$H$_5$).

6-[(*tert*-Butyloxycarbonyl)amino]-3-benzyl-1-hepten-5-one (10b). Compound **8b** (1.88 g, 3.98 mmol) was dissolved in CH$_3$CN (50 mL) and treated with *p*-toluenesulfonic acid monohydrate (832 mg, 3.98 mmol), and the resulting solution was kept at room temperature for 2 h. The solvent was removed, and the material was suspended in CH$_2$Cl$_2$ (50 mL). Di-*tert*-butyl dicarbonate (1.76 g, 7.96 mmol) and triethylamine (0.44 g, 4.4 mmol) were added, and the mixture was stirred at room temperature for 18 h. The solution was washed with 0.5 N HCl, H$_2$O, 1 M Na$_2$CO$_3$, and H$_2$O. The organic layer was dried over Na$_2$SO$_4$ and then evaporated to an oil. Flash chromatography on silica gel using MeCl$_2$ as the eluent gave 0.98 g (77.6%) of a colorless oil: NMR (CDCl$_3$) δ 1.36 (d, 3H, CH$_3$), 1.52 (s, 9H, *t*-Bu), 2.69 (m, 2H, CH$_2$C=O), 2.74 (m, 2H, CH$_2$Ar), 3.06 (m, 1H, C*H*CH=CH$_2$), 4.62 (m, 1H, C*H*NH), 4.16 (m, 3H, NH, vinyl), 5.82 (m, 1H, vinyl), 7.32 (m, 5H, C$_6$H$_5$).

7-Methyl-6-[(*tert*-butyloxycarbonyl)amino]-3-isopropyl-1-octen-5-one (10c). Compound **8c** (1.20 g, 2.73 mmol) was dissolved in CH$_3$CN (15 mL) and treated with *p*-toluenesulfonic acid monohydrate (860 mg, 4.5 mmol); the resulting solution was kept for 2 h. The mixture was concentrated under vacuum to an oil. This was dissolved in MeCl$_2$ (20 mL) and treated with di-*tert*-butyl dicarbonate (1.30 g, 6.0 mmol) followed by triethylamine (0.84 mL, 6.0 mmol). The reaction was stirred overnight and then evaporated to a yellow oil. Filter-pad chromatography using a step gradient of CCl$_4$ and MeCl$_2$ was used to obtain 589 mg (72%) of product as a clear syrup: MS *m/e* 298 (M + H$^+$); NMR (CDCl$_3$) δ 0.81 (m, 12H, CH$_3$), 1.40 (s, 9H, *t*-Bu), 1.46 (m, 2H, CHMe$_2$), 2.08 (m, 1H, C*H*CH=CH$_2$), 2.47 (m, 2H, CH$_2$C=O), 4.16 (m, 1H, C*H*NH), 4.96 (m, 3H, NH, vinyl), 5.55 (m, 1H, vinyl); ^{13}C-NMR (CDCl$_3$) 208.48, 208.86 (C=O isomers). Anal. Calcd for C$_{17}$H$_{31}$NO$_3$: C, H, N.

6-Methyl-5-[(*tert*-butyloxycarbonyl)amino]-4-oxo-2-benzylheptanoic Acid (11a). The *N*-BOC olefinic ketone **10a** (0.27 g, 0.78 mmol) in 10 mL of acetone was cooled to 0 °C and treated with a 0 °C solution of 0.90 g (4.2 mmol) of NaIO$_4$ and 10.5 mg of RuO$_2$·*x*H$_2$O (59.27% Ru) in 5 mL of H$_2$O. The mixture was stirred at room temperature for 1 h and then filtered through Celite, followed by washing with acetone. The filtrate and washes were combined and saturated with NaCl. The biphasic mixture was extracted with CHCl$_3$ (filtering of emulsions was necessary). The organic phase was washed with 10% NaHSO$_3$, dried over Na$_2$SO$_4$, and concentrated to yield the product as a light brown foamy solid: 0.18 g (64%); MS *m/e* 364 (M + H). Anal. Calcd for C$_{20}$H$_{20}$NO$_5$: C (0.5), H, N.

5-[(*tert*-Butyloxycarbonyl)amino]-4-oxo-2-benzylhexanoic Acid (11b). An aliquot (4 mL) of a solution containing NaIO$_4$ (4.86 g, 22.6 mmol) in H$_2$O (20 mL) was used to dissolve RuO$_2$·*x*H$_2$O (6.4 mg, 59.3% Ru). A second aliquot (4 mL) of the metaperiodate solution was added to a solution of compound **10b** (900 mg, 2.84 mmol) in acetone (30 mL), and the resulting mixture was stirred at room temperature. The ruthenium−metaperiodate solution was added dropwise to the mixture over a 5-min period. The remaining periodate solution was then added dropwise over a 10-min period, and the

reaction mixture was stirred at room temperature for 3 h. The mixture was filtered through a pad of Celite and the pad washed with acetone (2 × 30 mL). The acetone was removed under vacuum and the resulting aqueous solution diluted with H$_2$O (80 mL) and saturated with NaCl. The aqueous medium was extracted with CH$_2$Cl$_2$ (3 × 100 mL), and the extracts were dried over Na$_2$SO$_4$ and evaporated *in vacuo* to a brown gum. Flash chromatography on silica gel using 4% MeOH in CHCl$_3$ afforded 603 mg (63.3%) of an oil: TLC R_f 0.32 (5% MeOH in CHCl$_3$); NMR (CDCl$_3$) δ 1.40 (m, 12H, CH$_3$), 2.65 (m, 1H, C*H*COOH), 2.90 (m, 2H, CH$_2$C=O), 3.27 (m, 2H, CH$_2$Ph), 4.28 (m, 1H, C*H*NH), 5.02 (bs, 1H, NH), 7.33 (m, 5H).

6-Methyl-5-[(*tert*-butyloxycarbonyl)amino]-4-oxo-2-isopropylheptanoic Acid (11c). This compound was prepared in 89% yield from **10c** in a manner very similar to that for **11b** above. The material was obtained as a solid: MS *m/e* 316 (M + H); NMR (CDCl$_3$) δ 0.79 (m, 12H, Me), 1.34 (s, 9H, *t*-Bu), 2.02 (m, 2H, C*H*Me$_2$), 2.45 (m, 1H, C*H*COOH), 2.72 (m, 2H, CH$_2$C=O), 4.20 (m, 1H, C*H*NH), 5.16 (d, 1H, NH). Anal. Calcd for C$_{16}$H$_{29}$NO$_4$: C (0.5), H, N.

α-(Carbobenzyloxy)nitroarginyl-ε-(carbobenzyloxy)lysylaspartic Acid β-Benzyl Ester (16). β-Benzyl-α-benzhydrylaspartate *p*-toluenesulfonate salt (**14**) was prepared by treatment of the tosylate salt of β-benzyl asparate in CHCl$_3$ with 1.1 equiv of diphenyldiazomethane at 0 °C (purple color discharged) followed by removal of solvent *in vacuo* and washing with Et$_2$O. A mixture of 6.67 g (10.85 mmol) of α,ε-dicarbobenzoxynitroarginyllysine (**13**),[20] 1.25 g (10.85 mmol) of *N*-hydroxysuccinimide, and 200 mL of THF was cooled to −10 °C and treated with 2.46 g (11.93 mmol) of DCC. The mixture was stirred at ambient temperature for 18 h and treated with 6.09 g (10.85 mmol) of the aspartic diester (**14**) in 60 mL of THF containing 1.6 mL (11.5 mmol) of triethylamine. The mixture was stirred for 4 h and filtered and the filtrate concentrated *in vacuo* to leave 9.4 g of a white foam. Chromatography on silica gel (3% MeOH in MeCl$_2$) afforded 7.29 g (68%) of the blocked tripeptide ester (**15**). The diester was treated with an ice-cold solution of 10 mL of anisole in 75 mL of TFA. After 30 min at 0° the solution was evaporated *in vacuo* and the residue triturated with 400 mL of Et$_2$O. The mixture was filtered and the white solid washed repeatedly with Et$_2$O and dried to leave 5.30 g (87%) of product as a white powder: TLC R_f 0.2 free of diester (**15**) at R_f 0.8 (CHCl$_3$−MeOH−H$_2$O; 8:3:1, lower phase). Anal. Calcd for C$_{39}$H$_{48}$N$_8$O$_{12}$: C, H, N.

6-[[α-(Carbobenzyloxy)nitroarginyl-ε-(carbobenzyloxy)lysylaspartyl(β-benzyl ester)]amino]-7-methyl-3-benzyl-1-octen-5-one (17). The blocked tripeptide (**16**, 2.2 g, 2.7 mmol) in 50 mL of THF was treated with 310 mg (2.7 mmol) of *N*-hydroxysuccinimide and 618 mg (3.0 mmol) of DCC. The mixture was stirred for 2.5 h when a solution of 1.13 g (2.7 mmol) of the amino ketone tosylate salt (**9a**) in 20 mL of THF containing 0.42 mL (3.0 mmol) of triethylamine was added. The resulting mixture was stirred for 3 h and the solvent removed *in vacuo*. The residue was partitioned between EtOAc (25 mL) and H$_2$O (25 mL) and the EtOAc phase dried over Na$_2$SO$_4$ and evaporated *in vacuo*. The residue was chromatographed on silica gel (3% MeOH in CHCl$_3$) to afford 2.45 g (88%) of product as a white powder: NMR (DMSO-d_6) δ 0.78 (t, 6H, CH$_3$), 1.4 (m, 10H, CH$_2$), 2.15 (br. q, 1H, C*H*Me$_2$), 2.65 (m, 5H, CH$_2$CO, C*H*CH=CH$_2$), 2.85 (m, 2H, C*H*$_2$C$_6$H$_5$), 3.05 (m, 2H, C*H*$_2$NH Cbz), 3.22 (m, 2H, C*H*$_2$NHC=N), 4.20 (m, 3H, NC*H*CO), 4.85 (m, 2H, olefin), 5.15 (m, 6H, CBZ, OCH$_2$Ph), 5.60 (m, 1H, olefin), 7.35 (m, 20H, C$_6$H$_5$). Anal. Calcd for C$_{55}$H$_{69}$N$_9$O$_{12}$: C, H, N.

5-[[α-(Carbobenzyloxy)nitroarginyl-ε-(carbobenzyloxy)lysylaspartyl(β-benzyl ester)]amino]-6-methyl-3-benzyl-4-oxoheptanoic Acid (18). A solution of the olefinic ketopeptide (**17**, 3.15 g, 3 mmol) in 200 mL of acetone was cooled to 0 °C and treated with a solution of 5.13 g (24 mmol) of NaIO$_4$ in 45 mL of H$_2$O containing 50 mg of RuO$_2$·H$_2$O. The mixture was stirred for 2 h, filtered through Celite, and washed with acetone. The filtrate was evaporated to remove acetone and the aqueous residue extracted with CHCl$_3$ (3 × 20 mL). The CHCl$_3$ extract was washed with brine, dried over Na$_2$SO$_4$, and evaporated to leave a solid residue. The material was chro-

matographed on silica gel (5% MeOH in CHCl₃) to give 1.20 g (38%) of a white powder: NMR (DMSO-d_6) δ 0.87 (t, 6H, CH₃), 1.52 (m, 10H, CH₂), 2.16 (m, 1H, C*H*Me₂), 2.80 (m, 5H, CH₂-CO, CHCOO, CH₂COO), 3.30 (m, 4H, CH₂N), 4.05 (m, 3H, NCHCO), 5.03, 5.05, 5.10 (each s, each 2H, CH₂NCBZ, OCH₂-Ph), 7.29 (m, 20H, C₆H₅). Anal. Calcd for C₅₄H₆₇N₉O₁₄·H₂O: C, H, N (0.6).

Attachment of N-BOC Pseudodipeptides to Merrifield Chloromethyl Resin (19a–c). Isomer A of the ketomethylene dipeptide acid (11a) (0.500 g, 1.38 mmol) was dissolved in 50 mL of 90% MeOH. Solid CsHCO₃ (0.268 g, 1.38 mmol) was added and the mixture stirred at room temperature for 1 h. The solvent was removed under reduced pressure, and the resulting residue was dried by evaporation of toluene three times (50 mL each) and then placed under high vacuum overnight. The ketomethylene cesium salt was dissolved in DMF (50 mL), and Merrifield chloromethyl resin (2.01 g, 0.75 meq/g resin) was added to the solution. The mixture was stirred under an argon atmosphere at 50 °C for 48 h. The reaction was cooled to room temperature and filtered and the resin washed exhaustively with MeOH, MeCl₂, and 2-PrOH. The resin was rinsed with Et₂O and dried under vacuum for 24 h. This provided the desired derivatized resin 19a (isomer A) (2.29 g, 0.34 mequiv/g resin). Unreacted 11a (0.110 g) was recovered from the washes.

Isomer B of 11a (1.60 g, 4.41 mmol) was treated with CsHCO₃ (0.857 g, 4.42 mmol) in the same manner as that described above. Coupling to Merrifield chloromethyl resin (9.02 g, 0.75 mequiv/g resin) yielded the desired derivatized resin 19a (isomer B) (10.2 g, 0.32 mequiv/g resin).

Following the above procedure, pseudodipeptide acid (11b, 570 mg) was attached to resin (3.40 g) to afford 3.90 g (0.38 mequiv/g resin) of 19b. The pseudodipeptide (11c, 425 mg) was similarly attached to resin (2.62 g) to yield 2.89 g (0.30 mequiv/g) of 19c.

N^α-[(Butyloxycarbonyl)-N^ε-(2-chlorobenzyloxycarbonyl)-L-lysyl]-L-aspartic Acid β-Cyclohexyl Ester (20). N^α-(Butyloxycarbonyl)-N^ε-(2-chlorobenzyloxycarbonyl)-L-lysine (2.08 g, 5.0 mmol) and N-hydroxysuccinimide (0.634 g, 5.5 mmol) were dissolved in MeCl₂ (25 mL) and cooled to 0 °C. Dicyclohexylcarbodiimide (1.256 g, 6.0 mmol) in 15 mL of MeCl₂ was added dropwise over a 5-min period. The reaction mixture was stirred at 0 °C for 3 h and then stored at 4 °C overnight. The mixture was filtered and the filtrate evaporated to a solid. The trifluoroacetate salt of aspartic acid β-cyclohexyl ester [prepared by reacting N^α-BOC-aspartic acid β-cyclohexyl ester (2.21 g, 7.0 mmol) with 50% TFA in MeCl₂ (40 mL) for 1 h and evaporating the mixture to a solid] was dissolved in 50% aqueous THF, and the pH was adjusted to 8 with N-methylmorpholine. The succinimidyl ester was dissolved in THF (10 mL) and added to the aspartic acid solution. The pH was adjusted to 8 using N-methylmorpholine, and the reaction was allowed to stir overnight. The THF was removed under reduced pressure and the crude material partitioned between Et₂O (200 mL) and 5% NaHCO₃ (200 mL). The bicarbonate layer was acidified carefully to pH 3 and extracted with three 100-mL portions of EtOAc. The combined organic layers were dried over Na₂SO₄, filtered, and evaporated to a solid foam. The material was purified using silica gel and filter-pad chromatography—elutions were performed utilizing a step gradient of 0–10% EtOH in EtOAc containing 0.25% HOAc. Pure fractions were pooled and evaporated to give a solid foam: 2.33 g (76%); TLC R_f 0.23 (5% EtOH, 0.25% AcOH in EtOAc); NMR (CDCl₃) δ 1.60 (m, 19H, t-Bu, cyclohexyl), 2.87 (m, 2H, CH₂CO), 3.18 (m, 2H, CH₂NH), 4.18 (m, 1H, NCHCO), 4.75 (m, 3H, NH, OC*H*, NHC*H*C=O), 5.20 (s, 2H, ArCH₂), 5.42 (m, 1H, NH), 7.35 (m, 4H, Ar). Anal. Calcd for C₂₉H₄₂N₃O₉-Cl: C, H, N.

N^α-(tert-Butyloxycarbonyl)-L-prolyl-L-aspartic Acid β-Benzyl Ester (21). The procedure was analogous to the dipeptide preparation described above. BOC-L-proline succinimide (1.56 g, 5 mmol) was reacted with aspartic acid β-benzyl ester (1.33 g, 6 mmol) to give 1.20 g (57%). Anal. Calcd for C₂₁H₂₈N₂O₇: C, H, N.

N^α-(tert-Butyloxycarbonyl)-L-norleucyl-L-aspartic Acid β-Benzyl Ester (22). By a procedure analogous to the dipeptide preparation previously described, BOC-L-norleucine succinimide (3.90 g, 11.8 mmol) was reacted with aspartic acid β-benzyl ester (2.68 g, 12 mmol) to give 3.30 g (66%). Anal. Calcd for C₂₂H₃₂O₇N₂: C, H, N.

N^α-(Butyloxycarbonyl)-L-leucyl-L-aspartic Acid β-Benzyl Ester (23). The succinimide ester (6.64 g, 19.7 mmol) similarly prepared was coupled with L-aspartic acid β-benzyl ester (4.39 g, 19.7 mmol) to give 8.6 g (100%) of an oil: NMR (CDCl₃) δ 0.88 (d, 6H, CH₃), 1.46 (s, 9H, t-Bu), 1.40–1.65 (m, 3H, C*H₂*C*H*Me₂), 2.98 (m, 2H, CH₂C=O), 4.10 (m, 1H, NCH-CO), 4.78 (m, 1H, NCHCO), 4.97 (bd, 1H, NH), 5.05 (s, 2H, CH₂Ar), 6.30 (bs, 1H, NH), 7.29 (s, 5H, Ar).

N^α-(Butyloxycarbonyl)-L-alanyl-L-aspartic Acid β-Benzyl Ester (24). The succinimide ester of N-BOC-alanine (6.06 g, 20.0 mmol) was coupled to L-aspartic acid β-benzyl ester (4.46 g, 20.0 mmol) to afford an oil which was triturated with hexane/Et₂O to give 2.13 g (27%) of a foam: NMR (CDCl₃) δ 1.32 (d, 3H, CH₃), 1.39 (s, 9H, t-Bu), 2.96 (m, 2H, CH₂CO), 4.14 (m, 1H, NCHC=O), 4.80 (m, 1H, NCHCO), 5.08 (s, 2H, ArCH₂), 5.79 (s, 1H, NH), 7.15 (d, 1H, NH), 7.29 (s, 5H, Ar).

N^α-(Butyloxycarbonyl)-N^ε-(2-chlorobenzyloxycarbonyl)-D-lysyl-L-aspartic Acid β-Benzyl Ester (25). N^α-BOC-N^ε-2-ClZ-D-lysine (1.66 g, 4.0 mmol) and N-hydroxysuccinimide (0.49 g, 4.2 mmol) were reacted as above to form the succinimide ester. The ester was coupled with L-aspartic acid β-benzyl ester (0.98 g, 4.4 mmol) to give 1.85 g (78%): TLC R_f 0.33 (6% MeOH, 0.3% AcOH in MeCl₂); NMR (CDCl₃) δ 1.42 (s, 9H, t-Bu), 1.55 (m, 6H, CH₂), 3.03 (m, 2H, CH₂CO), 3.16 (m, 2H, CH₂N), 4.23 (m, 1H, NCHCO), 4.92 (m, 1H, NCH₂-CO), 5.02 (s, 2H, CH₂Ar), 5.20 (s, 2H, CH₂Ar), 5.25 (bs, 1H, NH), 7.30 (m, 5H, Ar).

Solid Phase Synthesis of Pseudopentapeptides 26a–h. Arg-Lys-Asp-Val(k)Phe (1a Isomer A). Following the general procedure of Almquist et al.,[21] BOC-Lys(ClZ)Asp(cHex) (20) (1.22 g, 2.0 mmol) and hydroxybenzotriazole hydrate (HOBT) (0.310 g, 2.03 mmol) were dissolved in DMF (5 mL) diluted with MeCl₂ (5 mL) and cooled to 0 °C in an ice bath. DCC (0.446 g, 2.16 mmol) in MeCl₂ (10 mL) was added dropwise over 5 min. The reaction mixture was stirred in MeCl₂ for 15 min. During the activation of the dipeptide, ketomethylene-derivatized Merrifield resin (19a-A) (2.29 g, 0.437 mequiv/g resin) was treated with 40% TFA/10% anisole in MeCl₂ (5 min, then 30 min) to remove the BOC group. The peptide-resin was washed alternately with MeCl₂ and 2-PrOH to remove residual TFA. The activated dipeptide solution above was filtered and added to the peptide-resin along with diisopropylethylamine (0.35 mL, 2.0 mmol). The reaction was shaken at room temperature and determined to be complete (negative Kaiser test) after 5 h. The BOC peptide-resin (26a) was washed successively with MeCl₂ (3×), 2-PrOH (2×), and MeCl₂ (3×). The BOC group was removed by treatment of the peptide-resin with 40% TFA/10% anisole in methylene chloride (5 min, then 30 min), and the resin was washed to remove TFA. N^α-BOC-N^g-tosylarginine (27, 0.86 g, 2.0 mmol) was converted to its HOBT ester using HOBT (0.316 g, 2.06 mmol) and DCC (0.448 g, 2.17 mmol) following the same procedure as described above. The activated ester was added to the resin along with diisopropylethylamine (0.350 mL, 2.0 mmol), and the reaction vessel was shaken overnight. The reaction was checked for completion (negative Kaiser test), and the peptide-resin was successively washed with MeCl₂ (3×), 2-PrOH (2×), MeCl₂ (2×), and MeOH (2×). The material was dried under vacuum to give 3.04 g of dry peptide-resin (28a). The BOC group was removed followed by washing as described above. The peptide-resin was dried under vacuum for 3 h and then treated with anisole (3 mL) in anhydrous HF (30 mL) for 90 min at 0 °C. The HF/anisole was removed under vacuum, and the resin was washed with anhydrous Et₂O. Crude final product was extracted from the resin using 10% acetonitrile in water with 0.5% TFA (four 25 mL extractions). The material was partially evaporated to remove CH₃CN, frozen, and lyophilized. The product was purified by preparative HPLC using a 5–25% gradient of CH₃CN in water containing 0.1% TFA. Pure fractions were pooled, evaporated of CH₃CN, frozen, and lyophilized to afford 308 mg (31%) of

1a (isomer A): FAB-MS *m/e* 663 (M + H$^+$). Anal. Calcd for C$_{31}$H$_{50}$N$_8$O$_8$·3CF$_3$CO$_2$H: C, H, N, F.

Compound **1a** (isomer B) was prepared in 21% yield in a similar manner from **19a-B** and **20**: FAB-MS *m/e* 663 (M + H). Anal. Calcd for C$_{31}$H$_{50}$N$_8$O$_8$·3.25CF$_3$COOH·H$_2$O: C, H, N, F.

Compound **1a-A** was also obtained by hydrogenation of 0.90 g of **18** (isomer A) over 0.80 g of Pd in HOAc containing 0.1 N HCl for 24 h. Filtration of catalyst followed by evaporation of solvent and washing with Et$_2$O afforded 0.36 g (56%) of **1a** as the HCl salt, equal to **1a** above by HPLC. Anal. Calcd for C$_{31}$H$_{50}$N$_8$O$_8$·3HCl·2.5H$_2$O: C, H, N, Cl. Other pseudopentapeptides in this series (**1b**−**h**) were obtained by the resin method as described above for **1a**. The reactants and analytical data are recorded below for these compounds which were obtained in 17−22% yields.

Arg-Pro-Asp-Val(k)Phe. 1b-A from **19a-A** and **21**: FAB-MS *m/e* 632 (M + H); partial NMR (D$_2$O) δ 0.76 (d, 3H, CH$_3$), 0.88 (d, 3H, CH$_3$), 4.37 (m, 2H, NCHCO), 4.50 (dd, 1H, NCHCO), 4.67 (dd, 1H, NHCHCO), 7.30 (m, 5H, C$_6$H$_5$); ^{13}C-NMR (D$_2$O) 210.04 (ketone). Anal. Calcd for C$_{30}$H$_{45}$N$_7$O$_8$·2CF$_3$COOH·H$_2$O: C, H (0.6), N, F (0.8).

1b-B from **19a-B** and **21**: FAB-MS *m/e* 632 (M + H); partial NMR (D$_2$O) δ 0.74 (d, 3H, CH$_3$), 0.81 (d, 3H, CH$_3$), 4.23 (d, 1H, NCHCO), 4.34 (t, 1H, NCHCO), 4.46 (dd, 1H, NCHCO), 4.63 (dd, 1H, NCHCO), 7.26 (m, 5H, C$_6$H$_5$); ^{13}C-NMR (D$_2$O) 210.68 (ketone). Anal. Calcd for C$_{30}$H$_{45}$N$_7$O$_8$·2CF$_3$COOH·H$_2$O: C, H, N, F (0.5).

Arg-Nle-Asp-Val(k)Phe. 1c-A from **19a-A** and **22**; FAB-MS *m/e* 648 (M + H); partial NMR (D$_2$O) δ 0.74 (d, 3H, CH$_3$), 0.83 (t, 3H, CH$_3$), 0.86 (d, 3H, CH$_3$), 4.01 (t, 1H, NCHCO), 4.29 (t, 1H, NCHCO), 4.35 (d, 1H, NCHCO), 4.70 (dd, 1H, NCHCO), 7.28 (m, 5H, C$_6$H$_5$); ^{13}C-NMR (D$_2$O) 210.00 (ketone). Anal. Calcd for C$_{31}$H$_{49}$N$_7$O$_8$·2.3CF$_3$COOH: C, H, N, F.

1c-B from **19a-B** and **22**: FAB-MS *m/e* 648 (M + H); partial NMR (D$_2$O) δ 0.76 (d, 3H, CH$_3$), 0.82 (t, 3H, CH$_3$), 0.83 (d, 3H, CH$_3$), 4.01 (t, 1H, NCHCO), 4.23 (d, 1H, NCHCO), 4.29 (t, 1H, NCHCO), 4.70 (dd, 1H, NCHCO), 7.28 (m, 5H, C$_6$H$_5$); ^{13}C-NMR (D$_2$O) 210.59 (ketone). Anal. Calcd for C$_{31}$H$_{49}$N$_7$O$_8$·2.25CF$_3$COOH: C, H, N, F.

Arg-Leu-Asp-Val(k)Phe. 1d-A from **19a-A** and **23**; FAB-MS *m/e* 648 (M + H); partial NMR (D$_2$O) δ 0.74 (d, 3H, CH$_3$), 0.87 (d, 6H, CH$_3$), 0.90 (d, 3H, CH$_3$), 4.10 (t, 1H, NCHCO), 4.36 (m, 1H, NCHCO), 4.39 (m, 1H, NCHCO), 4.70 (m, 1H, NCHCO), 7.29 (m, 5H, C$_6$H$_5$); ^{13}C-NMR (D$_2$O) 211.00 (ketone). Anal. Calcd for C$_{31}$H$_{49}$N$_7$O$_8$·1.75CF$_3$COOH: C, H, N, F.

Arg-Ala-Asp-Val(k)Phe. 1e-A from **19a-A** and **24**; FAB-MS *m/e* 606 (M + H); partial NMR (D$_2$O) δ 0.75 (d, 3H, CH$_3$), 0.86 (d, 3H, CH$_3$), 1.37 (d, 3H, CH$_3$), 4.00 (t, 1H, NCHCO), 4.36 (m, 2H, NCHCO), 4.67 (dd, 1H, NCHCO), 7.28 (m, 5H, C$_6$H$_5$); ^{13}C-NMR (D$_2$O) 211.16 (ketone). Anal. Calcd for C$_{28}$H$_{43}$N$_7$O$_8$·2CF$_3$COOH·2H$_2$O: C, H, N, F.

Arg-D-Lys-Asp-Val(k)Phe. 1f-A from **19a-A** and **25**: FAB-MS 663 (M + H); partial NMR (D$_2$O) δ 0.76 (d, 3H, CH$_3$), 0.86 (d, 3H, CH$_3$), 4.00 (t, 1H, NCHCO), 4.23 (t, 1H, NCHCO), 4.36 (dd, 1H, NCHCO), 7.27 (m, 5H, C$_6$H$_5$); ^{13}C-NMR (D$_2$O) 211.29 (ketone). Anal. Calcd for C$_{31}$H$_{50}$N$_8$O$_8$·2.85CF$_3$COOH·2H$_2$O: C (0.6), H, N, F (0.5).

Arg-Nle-Asp-Ala(k)Phe. 1g-A and **1g-B** from **19b** and **22**. The crude pseudopentapeptide obtained (80%) by cleavage from the resin was purified by preparative HPLC (24% CH$_3$-CN·0.1% TFA) to afford **1g-A** (21%) [FAB-MS *m/e* 620 (M + H). Anal. Calcd for C$_{29}$H$_{45}$N$_7$O$_8$·2CF$_3$COOH·H$_2$O: C, H, N] and **1g-B** (19%) [FAB-MS *m/e* 620 (M + H). Anal. Calcd for C$_{29}$H$_{45}$N$_7$O$_8$·2CF$_3$COOH·2H$_2$O: C, H, N].

Arg-Nle-Asp-Val(k)Val. 1h-A and **1h-B** from **19c** and **22**. The crude pseudopentapeptide (86%) was purified by HPLC (16−30% CH$_3$CN−0.1% TFA gradient elution) to give **1h-A** (18%) and **1h-B** (17%). **1h-A**: FAB-MS *m/e* 600 (M + H); partial NMR (D$_2$O) δ 0.77 (d, 3H, CH$_3$), 0.83 (m, 3H, CH$_3$), 0.89 (m, 9H, CH$_3$), 4.01 (t, 1H, NCHCO), 4.30 (t, 1H, NCHCO), 4.44 (d, 1H, NCHCO); ^{13}C-NMR (D$_2$O) 211.81 (ketone). Anal. Calcd for C$_{27}$H$_{49}$N$_7$O$_8$·2CF$_3$COOH: C, H, N, F. **1h-B**: FAB-MS *m/e* 600 (M + H); partial NMR (D$_2$O) δ 0.90 (m, 15H, CH$_3$), 4.04 (t, 1H, NCHCO), 4.32 (t, 1H, NCHCO), 4.33 (d, 1H,

NCHCO); ^{13}C-NMR (D$_2$O) 212.88 (ketone). Anal. Calcd for C$_{27}$H$_{49}$N$_7$O$_8$·2.2CF$_3$COOH·H$_2$O: C, H, N, F.

N-Acetyl-Arg-Pro-Asp-Val(k)Phe (1j-A). Resin-bound *N*-BOC pentapeptide (**28b-A**, from preparation of **1b-A**, 994 mg) was treated with 40% TFA/10% anisole in MeCl$_2$ (5 and 30 min) to remove the BOC group and the resin washed with MeCl$_2$ and 2-PrOH, followed by drying *in vacuo*. The dried resin (994 mg, as TFA salt) was neutralized with 5% diisopropylethylamine in MeCl$_2$ and thrice washed with MeCl$_2$. The resin was then treated with 2 mL of Ac$_2$O and 0.25 mL of pyridine in 8 mL of MeCl$_2$ for 2 h. The resin was washed with MeCl$_2$ and dried to leave 960 mg of *N*-acetyl resin-bound product (**29j**). The material was treated with dry HF−anisole (9:1) for 1.5 h at 0 °C to cause cleavage from the resin and removal of blocking groups. The mixture was evaporated *in vacuo* and the peptide extracted into 10% CH$_3$CN containing 0.5% TFA. Lyophilization afforded 231 mg of crude material, which was purified by preparative HPLC (14−20% CH$_3$CN gradient containing 0.1% TFA to afford 62 mg (98% pure) and 75 mg (90% pure): FAB-MS *m/e* 674 (M + H). Anal. Calcd for C$_{32}$H$_{47}$N$_7$O$_9$·1.25CF$_3$COOH·H$_2$O: C, H, N, F.

N-Acetyl-Arg-Nle-Asp-Val(k)Phe (1k-A). In a manner similar to that described for **1j**, the resin-bound intermediate **28c** (from preparation of **1c-A**) was treated with TFA in anisole to remove the BOC group and the free amine was acetylated with Ac$_2$O. The resulting resin-bound blocked peptide (**29k**) was treated with HF−anisole to provide crude **1k-A**, which was purified by HPLC: FAB-MS *m/e* 690 (M + H). Anal. Calcd for C$_{33}$H$_{51}$N$_7$O$_9$·CF$_3$COOH·H$_2$O: C, H, N, F.

Tris(benzyloxycarbonyl)-L-arginyl-*N*-methylnorleucine *tert*-Butyl Ester (31). A mixture of 2.45 g (10 mmol) of *N*-methylnorleucine, 1.5 mL of concentrated H$_2$SO$_4$, and 40 mL of dioxane was stirred for 15 min. Liquid isobutylene (30 mL) was added slowly to the mixture using a Dewar condensor with dry ice. The mixture was stirred for 5 h, poured into ice-cold 1 N NaOH (200 mL), and extracted with Et$_2$O (3 × 100 mL). The combined Et$_2$O extracts were washed with saturated NaHCO$_3$ (100 mL) and brine (2 × 100 mL), dried over MgSO$_4$, and evaporated to leave 1.05 g (52%) of *N*-Me-norleucine *tert*-butyl ester (**30**). A solution of *N*^{α},*N*^{δ},*N*^{ω}-tris(benzyloxycarbonyl)-L-arginine (2.88 g, 5.0 mmol) in MeCl$_2$ (50 mL) was cooled to −10 °C, and Et$_3$N (0.68 mL, 5.0 mmol) and isobutyl chloroformate (0.78 mL, 6.0 mmol) were added successively. After the reaction mixture was stirred for 20 min, a solution of 1.05 g of **30** in MeCl$_2$ (20 mL) was added, followed by Et$_3$N (0.68 mL, 5.0 mmol). The reaction mixture was allowed to come to room temperature and stirred for 4 h. The material was evaporated to an oil and subjected to flash chromatography using a step gradient of 10, 20, and 30% ethyl acetate in hexane (500 mL each), yielding 2.56 g (33.7%) of **31**: MS *m/e* 760 (M + H$^+$); ^{1}H-NMR (CDCl$_3$) δ 0.90 (t, 3H, CH$_3$), 1.01−1.95 (m, 10H, CH$_2$); 1.39 (s, 9H, *t*-Bu), 2.83 (s, 3H, NCH$_3$), 4.00 (m, 2H, CH$_2$N), 4.62 (m, 1H, Arg-CH-N), 5.08 (s, 2H, CH$_2$O), 5.12 (s, 2H, CH$_2$O), 5.20 (s, 2H, CH$_2$O), 5.58 (d, 1H, NleCH-N), 7.23−7.45 (m, 15H, Ar), 9.33 (s, 2H, NH). Anal. Calcd for C$_{41}$H$_{53}$N$_5$O$_9$: C, H, N.

N^α,N^δ,N^ω-Tris(benzyloxycarbonyl)-L-arginyl-*N*-methyl-L-norleucine (32). Compound **31** (1.14 g, 1.5 mmol) was dissolved in a mixture of TFA (10 mL) and MeCl$_2$ (10 mL) and stirred for 40 min. The material was concentrated to an oil, diluted in MeCl$_2$ (50 mL), washed with H$_2$O (4 × 50 mL) and brine (50 mL), dried over MgSO$_4$, and evaporated to give 940 mg (89%) of a white solid: MS *m/e* 552 (M − Z − H$_2$O); ^{1}H NMR (CDCl$_3$) δ 0.88 (t, 3H, CH$_3$), 1.01−1.95 (m, 10H, CH$_2$), 2.86 (s, 3H, NCH$_3$), 3.83 (m, 2H, CH$_2$N), 4.72 (m, 1H, ArgCH-N), 5.0−5.3 (m, 6H, OCH$_2$), 5.96 (d, 1H, Nle-CH-N), 7.23−7.45 (m, 15H, Ar), 8.2−9.4 (bs, 3H, NH). Anal. Calcd for C$_{37}$H$_{45}$N$_5$O$_9$: C, H, N.

N^α,N^δ,N^ω-Tris(benzyloxycarbonyl)-L-arginylmethyl-L-norleucyl-L-aspartic Acid β-Cyclohexyl-α-*tert*-butyl Ester (34). Compound **32** (910 mg, 1.30 mmol) in MeCl$_2$ (10 mL) and HOBT (213 mg, 1.40 mmol) in DMF (1 mL) were combined and cooled to −10 °C. Dicyclohexylcarbodiimide (310 mg, 1.50 mmol) in MeCl$_2$ (5 mL) was added dropwise over 5 min. The reaction mixture was stirred for 20 min at −10 °C and then at room temperature for 20 min. Aspartic acid β-cyclohexyl-

α-*tert*-butyl ester (**33**) (433 mg, 1.60 mmol) in MeCl$_2$ (10 mL) was added to the reaction, along with *N*-methylmorpholine (0.18 mL, 1.60 mmol), and stirred overnight. The mixture was evaporated to an oil and subjected to flash chromatography using a step gradient of 10, 20, and 30% EtOAc in hexane (500 mL each). Product fractions were pooled and evaporated to give 1.04 g (85%) of a white solid: MS *m/e* 957 (M + H$^+$); NMR (CDCl$_3$) δ 0.88 (t, 3H, CH$_3$), 1.0−2.0 (m, 20H, CH$_2$),; 1.47 (s, 9H, *t*-Bu), 2.65−2.90 (m, 5H, NCH$_3$, CH$_2$COO), 3.97 (m, 2H, CH$_2$N), 4.4−4.8 (m, 3H, OCH, ArgCH-N, NH), 5.08 (s, 2H, OCH$_2$), 5.12 (s, 2H, OCH$_2$), 5.20 (s, 2H, OCH$_2$), 5.60 (d, 1H, NleCH-N), 6.73 (d, 1H, Asp-CH-N), 7.20−7.48 (m, 15H, Ar), 9.30 (bs, 2H, NH). Anal. Calcd for C$_{51}$H$_{68}$N$_6$O$_{12}$: C, H, N.

$N^{\alpha},N^{\beta},N^{\omega}$-Tris(benzyloxycarbonyl)-L-arginyl-*N*-methyl-L-norleucyl-L-aspartic Acid β-Cyclohexyl Ester (35). Compound **34** (960 mg, 1.00 mmol) was dissolved in a mixture of MeCl$_2$ (10 mL) and TFA (10 mL) and stirred for 60 min at room temperature. The material was concentrated to an oil, diluted in MeCl$_2$ (50 mL), washed with H$_2$O (4 × 50 mL), dried over MgSO$_4$, and evaporated to give 840 mg (93%) of product: NMR (CDCl$_3$) δ 0.87 (t, 3H, CH$_3$), 1.0−2.0 (m, 20H, CH$_2$), 2.70−2.90 (m, 5H, NCH$_3$, CH$_2$CO), 3.96 (m, 2H, CH$_2$N), 4.50−4.85 (m, 3H, OCH, ArgCH-N, NH), 5.08 (s, 2H, OCH$_2$), 5.14 (s, 2H, OCH$_2$), 5.20 (s, 2H, OCH$_2$), 5.70 (d, 1H, NleCH-N), 5.95 (d, 1H, AspCH-N), 7.23−7.45 (m, 15H, Ar), 9.35 (bs, 2H, NH).

Solid Phase Synthesis of 6-Methyl-5(*S*)-[(L-arginyl-N^{α}-methyl-L-norleucyl-L-aspartyl)amino]-4-oxo-2-isopropylheptanoic Acid (Isomer A). Arg-NMeNle-Asp-Val(k)Phe (1l). Using the procedure described previously for the synthesis of Arg-Lys-Asp-Val(k)Phe (**1a**), the BOC group was removed from the BOC-Val(k)Phe-resin (**19a**, 0.50 g, 0.25 mequiv) by treatment with a cocktail containing 40% TFA and 10% anisole in MeCl$_2$ for 5 min, followed by a second treatment with fresh cocktail for 30 min. After removal of the BOC group, the resin was washed four times alternately with MeCl$_2$ and 2-PrOH and then finally with MeCl$_2$ (3×). Meanwhile, tripeptide **35** (675 mg, 0.75 mmol) and HOBT (122 mg, 0.80 mmol) were dissolved in DMF (1 mL), diluted with MeCl$_2$ (10 mL), and cooled to 0 °C (ice bath). Dicyclohexylcarbodiimide (186 mg, 0.90 mmol) was added and the resulting mixture stirred at 0 °C for 25 min and then at room temperature for 30 min. The solution was added to the resin along with diisopropylethylamine (140 µL, 0.80 mmol), and the resulting mixture was shaken overnight. The coupling was judged complete by using the Kaiser test. The resin was washed with MeCl$_2$ and 2-PrOH and then dried to leave 0.66 g of blocked resin-bound pentapeptide (**36**).

The peptide was cleaved from the resin with concomitant removal of side-chain protecting groups by treating the peptide-resin with 10% anisole in HF (10 mL) at 0 °C for 90 min, followed by vacuum distillation of the HF−anisole mixture. The resin was washed with Et$_2$O and the peptide extracted with 15% MeCN containing 0.5% TFA. Lyophilization of the extracts gave 92 mg of crude peptide. The product was purified by HPLC using a preparative gradient of 10−30% MeCN with 0.1% TFA present. Fractions containing pure compound were pooled, evaporated of MeCN, and lyophilized to give 21 mg of **1l** (isomer A): HPLC 98% pure, 27% MeCN containing 0.1% TFA; FAB-MS *m/e* 662 (M + H$^+$); partial NMR (D$_2$O) δ 0.76 (t, 3H, CH$_3$CH), 0.86 (m, 6H, CH$_3$), 3.02 (1s, 3H, NCH$_3$), 4.33 (m, 1H, NCHCO), 4.52 (m, 1H, NCHCO), 4.58 (m, 1H, NCHCO), 4.90 (m, 1H, NCHCO), 7.20−7.38 (m, 5H, C$_6$H$_5$). Anal. Calcd for C$_{32}$H$_{51}$N$_7$O$_8$·2CF$_3$CO$_2$H: C, H, N.

Arg-NMeNle-Asp-Val(k)Val (1m). Using the procedure described for the preparation of **1l** above the BOC group was removed from 1.0 g of the BOC pseudodipeptide-resin unit **19c**. The resultant amino ketone was coupled with the blocked tripeptide (**35**, 333 mg), followed by a second treatment with 110 mg of **35** to afford 1.32 g of blocked pseudopentapeptide, **37**. Following cleavage from the resin the material was extracted into 50% HOAc and evaporated to leave 255 mg of crude pseudopentapeptide. Purification by preparative HPLC (16−27% MeCN−0.1% TFA) yielded 55 mg of **1m-A** and 41 mg of **1m-B**.

1m-A: FAB-MS *m/e* 614 (M + H); partial NMR (D$_2$O) δ 0.78 (t, 3H, CH$_3$), 0.85 (m, 3H, CH$_3$), 0.91 (m, 9H, CH$_3$), 3.03 (br s,

3H, NCH$_3$), 4.44 (m, 1H, NCHCO), 4.58 (m, 1H, NCHCO), 4.74 (dd, 1H, NCHCO), 4.93 (dd, 1H, NCHCO).

1m-B: FAB-MS *m/e* 614 (M + H); partial NMR (D$_2$O) 0.80 (d, 3H, CH$_3$), 0.88 (t, 3H, CH$_3$), 0.93 (m, 9H, CH$_3$), 3.06 (s, 3H, NCH$_3$), 4.49 (d, 1H, NCHCO), 4.56 (t, 1H, NCHCO), 4.73 (dd, 1H, NCHCO), 4.91 (dd, 1H, NCHCO).

***N*-(L-Arginyl-L-norleucyl-L-aspartyl)-5-amino-6-methyl-4-hydroxy-2-benzylheptanoic Acid Lactone (38).** Isomer A of **1c** (78 mg, 90 µmol) in MeOH (1.0 mL) was treated dropwise with 0.2 M LiBH$_4$ in THF (5.0 mL, 1.0 mmol). After 20 min, the reaction was judged complete by HPLC and the material evaporated twice from MeOH (25 mL each). HPLC analysis indicated a 19:1 ratio of open hydroxy acid to lactone. The material was twice purified on HPLC using a 15−26% gradient of MeCN containing 0.1% TFA. Under the acidic conditions, pure fractions of the product immediately began to lactonize. Fractions containing pure lactone were pooled, evaporated of MeCN, and lyophilized to give 54 mg (100% pure) of lactone: FAB-MS *m/e* 632 (M + H$^+$).

***N*-(L-Arginyl-L-norleucyl-L-aspartyl)-5-amino-6-methyl-4-hydroxy-2-benzylheptanoic Acid (1n-A).** The lactone **38** (8.8 mg, 10 µmol) was dissolved in H$_2$O (2 mL) and 1 N NaOH (40 µL, 40 µmmol) added. The saponification of the lactone ring was followed by analytical HPLC and was determined to be complete within 30 min. Carbon dioxide (gas) was bubbled through the solution until the pH was reduced to 8. The material was lyophilized to give isomer A of **1n** as the Na salt.

CEM Cell Binding Assays. CEM cells, a T-cell line, were obtained from the American Type Culture Collection (Rockville, MD). The cells were then subcultured in flasks at 2 × 10^5 cells per flask in RPMI-1640 in 20% serum. Cells were stored frozen at 3 × 10^6 cells/mL in vials prior to use. Binding assays were carried out according to a modification of the method of Audhya et al.[23] using [^{3}H]thymopentin in the presence of bacitracin. CEM cells were adjusted to a final concentration of 5 × 10^6 cells/0.1 mL of binding buffer (RPMI-1640 containing bacitracin). Dilutions of thymopentin or thymopentin analogs were added to the cells over a concentration range of 10^{-6}−10^{-3} M. [^{3}H]Thymopentin (specific activity of 37 Ci/mmol) was then added (548 000 cpm), and the tubes were incubated at 25 °C for 30 min. At the end of the incubation time, the cells were diluted with 1 mL of ice-cold binding buffer. The cells were washed two times (3 mL each wash) by centrifugation at 600*g*; the final pellets were resuspended and solubilized, and the radioactivity determined by scintillation counting.

Thymocyte Binding Assays. Binding was quantified using mouse thymocytes. Thymus cells were obtained by dissociation of the tissue and removal of adherent cells by glass wool chromatograpy. TP-5 binding was carried out as described by Amoscato et al.[24] with the addition of bacitracin to the culture medium and the omission of oil in the cell washing.

Determination of Serum Half-Life. Analysis of serum half-lives of the analogs was carried out using a modification of HPLC procedures described by Amoscato et al.[25] Briefly, the test compounds were incubated in heparinized human blood plasma at 37 °C for variable, specified time periods ranging from 0 to 30 min. Incubation in serum was terminated by addition of trifluoroacetic acid (TFA) to a final concentration of 10% (v/v). The mixture was maintained at 0 °C for 1 h, and the serum proteins were then removed by centrifugation (10000*g* for 15 min). The supernatants were collected and stored at −20 °C prior to HPLC analysis. HPLC fractionation was carried out using isocratic gradients of acetonitrile in water in the presence of TFA. Identification of TP-5, TP-5 analogs, and any degradation products was performed using peak elution times (absorbance 280 nm) to provide an index of time-dependent conversion between starting material and metabolite.

Mouse serum samples were prepared from whole blood using either heparin sodium or ethylenediaminetetraacetic acid (EDTA) as chemical anticoagulants. The use of two collection methods was to assess whether half-life measurements of the compounds might be altered by collection

methods, such protease inhibitor, i.e. by (EDTA) inhibitors serum proteases.

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