# Structure−Activity Relationships of Novel Hematoregulatory Peptides

Pradip K. Bhatnagar,*,† Erik K. Agner,‡ Doreen Alberts,† Bente E. Arbo,‡ James F. Callahan,† Allan S. Cuthbertson,‡ Steinar J. Engelsen,§ Hege Fjerdingstad,‡ Michael Hartmann,§ Dirk Heerding,† Johann Hiebl,§ William F. Huffman,† Mette Hysben,‡ Andrew G. King,⊥ Peter Kremminger,§ Chet Kwon,† Steve LoCastro,† Dagfinn Løvhaug,‡ Louis M. Pelus,⊥ Steve Petteway,⊥,|| and Joanne S. Takata†

*Departments of Medicinal Chemistry and Molecular Virology and Host Defense, SmithKline Beecham Pharmaceuticals, King of Prussia, Pennsylvania 19406, Nycomed Pharma As, Gaustadalléene 21, Oslo, Norway, and Hafslund Nycomed Pharma, St. Peter-Strasse 25, P.O. Box 120, A-4021 Linz, Austria*

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Hematopoiesis is a lifelong cell renewal process regulated by a family of lineage specific hematopoietic growth factors. Several hematopoietic growth factors such as G-CSF, GM-CSF, and M-CSF have been clinically evaluated for enhancement of host defense in normal and immunocompromised patients and for the treatment of infectious diseases. This paper reports the structure−activity relationships of low molecular weight hematoregulatory peptides based on a nonapeptide (**1**, SK&F 107647). Like the macromolecular growth factors, these peptides modulate host defense. A molecular target for this class of compounds has not yet been identified. However, the structure−activity relationships established by this study implicate a very specific molecular recognition event that is pivotal for the biological activities of **1** and its analogues.

## Introduction

In recent years considerable attention has been devoted to hematopoiesis, the phenomenon of differentiation and formation of mature blood cells.[1−3] Several hematopoietic growth factors, cytokines, and chemokines have been found to modulate formation of mature blood cells from unique stem cells.[4−7] Mature blood cells, such as neutrophils and monocytes, are the first line of defense against pathogens. Drugs that stimulate hematopoiesis or host defense mechanisms in normal and immune-compromised hosts can potentially act as novel antimicrobial agents, and several hematopoietic growth factors are currently being clinically evaluated for their antimicrobial activities.[8−10] Since these growth factors are large proteins and consequently may not be suitable for chronic use, an alternative approach would be the identification and development of small molecules that mimic the activities of these growth factors and modulate hematopoietic processes.

In 1984, Paukovits *et al.*[11] reported the hematopoietic inhibitory activity of a pentapeptide, pGlu-Glu-Asp-Cys-Lys. This peptide, termed HP-5, was isolated from mature human leukocytes. While HP-5 is a negative regulator of myelopoiesis, the facile oxidation of the cysteine residue yields a disulfide-linked dimer that stimulates hematopoiesis *in vitro*.[12] During our research with HP-5 dimer, we found that the biological activity of this peptide was extremely erratic. We considered that these difficulties might arise from the facile redox chemistry available to this peptide. To stabilize the oxidized peptide, we replaced the reducible disulfide bond with an isosteric ethylene spacer.[13] The resulting compound **1** (SK&F 107647, Figure 1) has potent hematoregulatory activity[14] and has been demonstrated to be efficacious in several animal models of infectious diseases and bone marrow transplantation.[15−18] *In vitro*, SK&F 107647 (**1**) stimulates murine and human primary stromal cultures, as well as stromal cell lines to produce colony-stimulating activity (CSA).[19] It is currently under clinical evaluation for infectious complications in neutropenic patients.

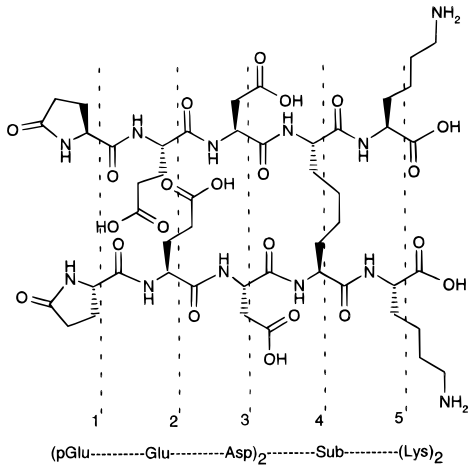


**Figure 1.** Structure and numbering system used for **1**.

Several structurally close analogues of compound **1** were synthesized and tested for their ability to induce CSA from a stromal cell line. The results reported here indicate that **1** and its analogues have stringent structural requirements for CSA activity. On the basis of these results, we infer that **1** probably interacts with an as yet unidentified cellular target in a very specific manner. This research has also led us to the discovery of compounds that are $10^3-10^4$ times more potent than the initial lead **1**.

## Chemistry

Peptides were synthesized using solid phase techniques.[20,21] The *N*-α-Boc protection scheme was used with either phenylacetamidomethyl (PAM) resin or benzhydrylamine (BHA) resin. Peptides were depro-

† Department of Medicinal Chemistry.
‡ Nycomed Pharma As.
§ Hafslund Nycomed Pharma.
⊥ Department of Molecular Virology and Host Defense.
|| Current address: Cato Research, 4364 S. Austin Ave., Durham, NC 27713.
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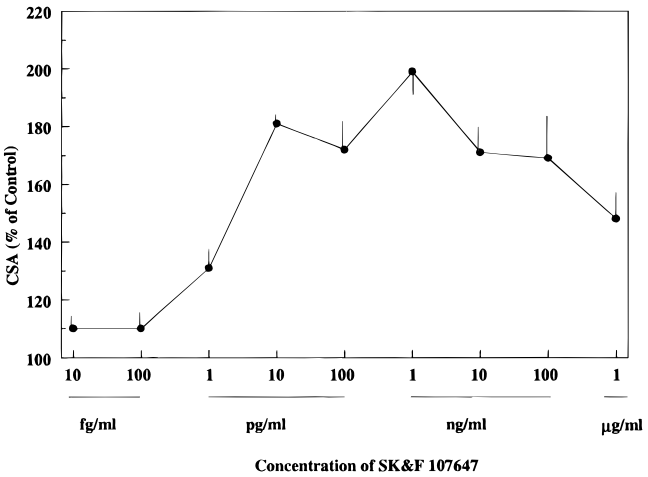



**Figure 2.** Composite dose−response analysis of SK&F 107647 on colony-stimulating activity induced from C6 stromal cells. CFU-GM numbers are presented as percentage of control ± standard error of the mean from 47 independent experiments.

tected and cleaved from the resin using anhydrous HF with anisole (9:1, v/v). Cleaved peptide-resin mixtures were washed with ether, and the peptides were extracted into 0.1% aqueous TFA and lyophilized.

The diaminosuberic and diaminoadipic acid derivatives were synthesized according to a previously described method.[22] The remaining diamino dicarboxylic acid derivatives were synthesized using methods developed by Schöllkopf *et al.*[23,29]

To ensure that both carboxyl groups of the diamino dicarboxylate derivatives reacted, the general method of coupling was modified.[13] Only 0.5 mol equiv of the diamino dicarboxylic acid derivative and 1 mol equiv of the coupling agents were used, and the coupling was allowed to proceed for 48 h. If the coupling was not complete after 48 h, as monitored by qualitative ninhydrin analysis,[20] an additional 1 mol equiv of the coupling reagents was added and coupling was allowed to proceed for an additional 24 h.

Crude peptides were purified by preparative reverse phase HPLC. Each peptide was assessed for purity by analytical HPLC and characterized by amino acid analysis and FAB mass spectrometry.

## Biology

Compound **1** exerts its colony-stimulating activity by inducing hematopoietic growth factors from human and murine stromal cell cultures.[24] The analogues of **1** were evaluated for induction of colony-stimulating activity in a standard murine granulocyte macrophage colony-forming unit (CFU-GM) assay.[25] In this assay, SK&F 107647 (**1**) and its active analogues exhibit a bell-shaped dose−response. A composite dose−response curve for **1** from over 47 experiments is shown in Figure 2. The functional cellular assay (CFU-GM) used to evaluate the CSA induced by the analogues is variable between experiments with respect to CFU-GM growth parameters, such as colony number, size, and maximal response to stromal cell supernate; however, variation within an experiment is minimal. These experimental variations made it difficult to directly compare the maximal colony-stimulating activity produced by these analogues when evaluated in separate assays. However, the $EC_{50}$ values estimated for each compound did not vary significantly for compounds tested in multiple experiments. The estimated $EC_{50}$ of each analogue was normalized to the $EC_{50}$ of **1** (from the ascending part of their respective dose−response curves) within a given set of experiments.

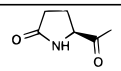
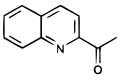
**Table 1.** Relative Potency for Induction of Colony-Stimulating Activity of C6 Stromal Line by Analogues Containing D-Amino Acid

| no. | structure | relative potency[a] |
|---|---|---|
| **1** | (pGlu-Glu-Asp)$_2$-Sub-(Lys)$_2$[b] | 1 |
| **2** | (D-pGlu-Glu-Asp)$_2$-Sub-(Lys)$_2$ | $1 \times 10^{-4}$ |
| **3** | (pGlu-D-Glu-Asp)$_2$-Sub-(Lys)$_2$ | $1 \times 10^{-5}$ |
| **4** | (pGlu-Glu-D-Asp)$_2$-Sub-(Lys)$_2$ | na[d] |
| **5** | (pGlu-Glu-Asp)$_2$-D,D-Sub-(Lys)$_2$[c] | na |

[a] Relative potency = [$EC_{50}$(**1**)]/[$EC_{50}$(compound)]. [b] Sub = (2*S*,7*S*)-2,7-diaminosuberic acid. [c] D,D-Sub = (2*R*,7*R*)-2,7-diaminosuberic acid. [d] na = relative potency < $1 \times 10^{-6}$.

**Table 2.** Relative Potency for Induction of Colony-Stimulating Activity of C6 Stromal Line by Position 1 and 5 Analogues

(Xxx-Glu-Asp)$_2$-Sub-(Lys)$_2$

| no. | Xxx | Relative Potency[a] |
|---|---|---|
| **1** |  | 1 |
| **6** |  | $1x10^{-3}$ |
| **7** |  | $1x10^{-3}$ |
| **8** |  | $1x10^{-4}$ |
| **9** |  | na[b] |
| **10** |  | na |
| **11** |  | na |
| **12** |  | na |

[a] Relative potency = [$EC_{50}$(**1**)]/[$EC_{50}$(compound)]. [b] na = relative potency < $1 \times 10^{-6}$.

## Results

To determine the structural requirements for activity, symmetrical substitutions of each amino acid at each position of **1** were studied. Substitution of any of the L-amino acids with their corresponding D-antipode results in much diminished activity[26] (Table 1). In addition, the deletion of any amino acid from the sequence resulted in the loss of ability to induce colony-stimulating activity from stromal cells (data not shown). These results suggest that the entire amino acid sequence of **1** with each residue in the L configuration is required for biological activity.

The pGlu (pyroglutamic acid) at position 1 can be replaced with selected cyclic carboxylic acids containing a nitrogen atom α to the carboxyl group (Table 2, **6**−**8**). While compared to **1** ($EC_{50}$ 1−5 pg/mL, 95% confidence

**Table 3.** Relative Potency for Induction of Colony-Stimulating Activity of C6 Stromal Line by Position 2, 3, 4, and 5 Analogues (Substitutions Shown in Bold)

| | structure | | | | | |
|---|---|---|---|---|---|---|
| no. | 1 | 2 | 3 | 4 | 5 | relative potency[a] |
| **1** | pGlu | Glu | Asp | Sub[c] | Lys | 1 |
| **13** | pGlu | **Asp** | Asp | Sub | Lys | 1 |
| **14** | pGlu | **Ser** | Asp | Sub | Lys | 10 |
| **15** | pGlu | Glu | **Glu** | Sub | Lys | 1 |
| **16** | pGlu | Glu | Asp | **cystine** | Lys | $10^{-2}$ |
| **17** | pGlu | Glu | Asp | **Adp**[d] | Lys | 1000 |
| **18** | pGlu | Glu | Asp | **Pim**[e] | Lys | na[b] |
| **19** | pGlu | Glu | Asp | **Aza**[f] | Lys | na |
| **20** | pGlu | Glu | Asp | **Asa**[g] | Lys | na |
| **21** | pGlu | Glu | Asp | Sub | D-**Lys** | na |
| **22** | pGlu | Glu | Asp | Sub | **Arg** | na |
| **23** | pGlu | Glu | Asp | Sub | **Orn** | na |
| **24** | pGlu | Glu | Asp | Sub | **Dha**[h] | na |
| **25** | pGlu | Glu | Asp | Sub | **Lys-NH$_2$** | 1 |
| **26** | pGlu | Glu | Asp | Sub | **lysinol** | na |

[a] Relative potency = [$EC_{50}$(**1**)]/[$EC_{50}$(compound)]. [b] na = relative potency < $1 \times 10^{-6}$. [c] Sub = (2*S*,7*S*)-2,7-diaminosuberic acid. [d] Adp = (2*S*,5*S*)-2,5-diaminoadipic acid. [e] Pim = (2*S*,6*S*)-2,6-diaminopimelic acid. [f] Aza = (2*S*,8*S*)-2,8-diaminoazelaic acid. [g] Asa = (2*S*,9*S*)-2,9-diaminosebacic acid. [h] Dha = (2*S*)-2,6-diamino-4-hexynoic acid.

range from 47 experiments), these analogues have much lower potency; however, they are still as efficacious (equivalent maximum CSA stimulation) as **1** and are potent hematopoietic compounds with $EC_{50}$'s in the nanomolar range. Other monocyclic carboxylic acids that lack a nitrogen at this position or polycyclic carboxylic acids (*e.g.*, quinaldic acid, 1-indole-2-carboxylic acid) are not tolerated.[27, 28]

The Glu at position 2 and Asp at position 3 were individually replaced with a set of amino acids (Asn, Asp, $\beta$-Asp, Gln, Glu, $\gamma$-Glu, His, Lys, Ser, Thr, Pro, Val, Phe, and Tyr) that have hydrophilic, hydrophobic, aromatic, positively charged, and negatively charged side chains. To test the necessity of an α-peptide linkage (rather that an isopeptide linkage) between residues 2 and 3 or 3 and 4, $\beta$-Asp and $\gamma$-Glu were also included in the set. The Glu to Ser substitution resulted in a 10-fold increase in the biological activity (ability to induce CSA from stromal cells). Surprisingly, Thr, which also contains a $\beta$-hydroxy group, was not an acceptable substitution at this position. The structural requirements were found to be even more stringent for position 3. The biological activity was only retained with the Glu substitution (Table 3, **17**) suggesting that interaction of this residue with some positively charged group was required for biological activity.

To study the requirements for position 4, several diamino dicarboxylic acids were synthesized and incorporated into the sequence. Analogues with more than four methylene units spanning the diamino dicarboxylic acids, *e.g.*, (2*S*,8*S*)-diaminoazelaic acid (**20**) and (2*S*,9*S*)-diaminosebacic acid (**21**),[29] were inactive at the doses tested. Analogue **19**, which contains (2*S*,6*S*)-diaminopimelic acid (three methylene units in the spacer), was also inactive. However, incorporation of (2*S*,5*S*)-diaminoadipic acid, a diamino dicarboxylic acid containing two methylene units in the spacer, at position 4 yielded an analogue (**18**) which was $10^3$ times more potent than **1**.

The lysine at position 5 was substituted with D-Lys, Arg, Orn, and (2*S*)-2,6-diamino-4-hexynoic acid (Dha) (Table 3, **21**−**24**). All of these substitutions resulted in the loss of biological activity. Substitution of $Lys^5$ with various other amino acids also yielded analogues that were inactive at the dose tested.[30] The C-terminal lysine carboxylates of **1** could be replaced by carboxamides (**25**) without any loss of biological activity; however, their deletion[30] or reduction to hydroxy methyl (**26**) groups was detrimental for biological activity (Table 3).

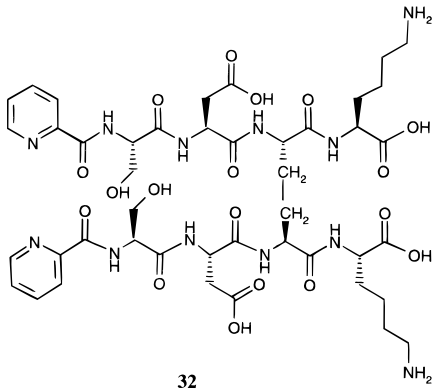
**Table 4.** Relative Potency for Induction of Colony-Stimulating Activity of C6 Stromal Line by Analogues with Multiple Amino Acid Substitutions

| no. | structure | relative potency[a] |
|---|---|---|
| **1** | (pGlu-Glu-Asp)$_2$-Sub-(Lys)$_2$ | 1 |
| **27** | (pGlu-Glu-Lys)$_2$-Sub-(Asp)$_2$ | na[b] |
| **28** | (Pic-Ser-Ala)$_2$-Sub-(Nle-NH$_2$)$_2$[c] | inactive |
| **29** | (Pic-Ser-Ser)$_2$-Adp-(Gln-NH$_2$)$_2$ | inactive |
| **30** | (Pic-Glu-Asp)$_2$-Adp-(Lys-NH$_2$)$_2$ | 100 |
| **31** | (Pic-Ser-Asp)$_2$-Sub-(Lys)$_2$ | 100 |
| **32** | (Pic-Ser-Asp)$_2$-Adp-(Lys)$_2$ | 10000 |

[a] Relative potency = [$EC_{50}$(**1**)]/[$EC_{50}$(compound)]. [b] na = relative potency < $1 \times 10^{-6}$. [c] Pic = picolinoyl.

Analogues **27**−**29** (Table 4) were synthesized to examine the importance of the overall charge and charge distribution of **1**. None of these analogues were active at the highest dose tested (1 $\mu$g/mL). This result, combined with the lack of activity of **27**, where the Asp and Lys residues were translocated, suggests that the relative location of positive and negative charge within **1** is critical for biological activity.

To investigate the effects of combining optimal substitutions at various positions of **1,** we prepared compounds **30**−**32**. While all of these compounds were active, compound **32**, which combines three of the best substitutions, is $10^4$ times more potent than the initial lead.



**32**

## Discussion

The data reported above demonstrate that the structure−activity relationships for SK&F 107647 are very stringent. There are few allowable substitutions that result in either comparable or enhanced biological activity. Most of the substitutions render the compound virtually inactive. D-Amino acid substitutions or amino acid truncations are detrimental for CSA-inducing activity. The pGlu at position 1 can be substituted with heterocyclic carboxylic acids that contain a nitrogen atom α to the carboxylic acid. The Glu at position 2 can be replaced with Asp or Ser, suggesting that a charged side chain is not required at this position. The lack of activity of Thr at this position suggests that either the spatial disposition of the $\beta$-hydroxyl group

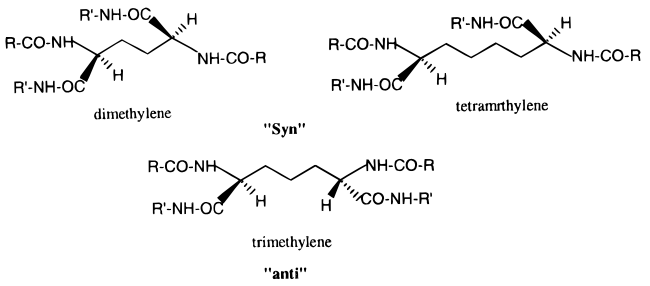



**Figure 3.** Extended conformations of spacers containing an odd or even number of carbon atoms.

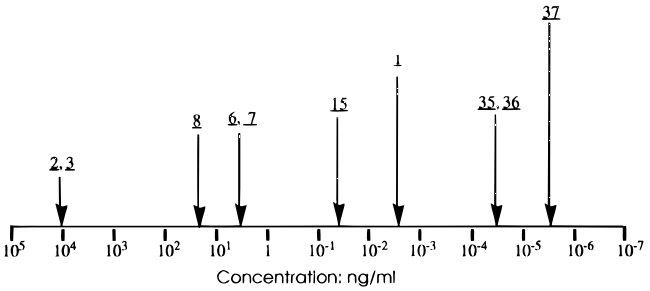



**Figure 4.** Range of $EC_{50}$'s of SK&F 107647 and its analogues in the C6 stromal cell CSA assay.

or the steric requirements at the $\beta$-carbon are critical. The stringent requirement for a carboxylic acid residue at position 3 suggests that this residue forms a critical salt bridge with some basic residue. The amino group of lysine at position 5 is also very important for the biological activity and may also be involved in an ionic interaction. The C-terminal carboxyl group of lysine can be replaced with the carboxamide without any loss of activity.

The number of methylene units spanning the diamino dicarboxylic acids at position 4 appears to be critical. The di- and tetramethylene spacers are well tolerated, whereas the mono-, tri-, penta-, and hexamethylene spacers are not. This suggests that both the length of the span and the relative conformation of the methylene units are important for biological activity. If we assume the bridge adopts an extended conformation, an even-membered alkylene spacer will place the two peptide chains in a "syn" relationship, while the odd-membered spacer will orient the peptide chains in an "anti" relationship (Figure 3). It is possible that this type of conformational bias plays a role in determining the biological activity of a given analogue. The results shown in Table 4 indicate that both the net charge and the exact location of the charged groups are critical for biological activity. Compound **27**, in which Asp at position 3 and Lys at position 5 were interchanged, was not active at the highest dose tested (1 $\mu$g/mL, *i.e.*, relative potency $< 10^{-6}$). The stringent structural requirements for this peptide and the availability of a panel of analogues with $EC_{50}$ values ranging from micromolar to femtomolar (Figure 4) strongly suggest that these compounds interact with a specific yet unidentified molecular target.

We have shown that **1** and its analogues can induce colony-stimulating activity from stromal cells. *In vivo*, **1** increases the proliferative rate of CFU-GM cell cycle and modulates effector cell functions.[13-18] These properties make **1** and its analogues a remarkable class of compounds that act through host defense modulation. A greater understanding of their mechanism of action and required structural parameters for biological activity may allow us to design novel therapeutic agents.

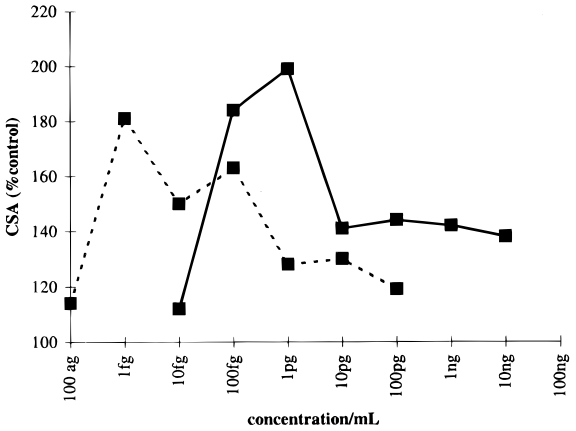



**Figure 5.** Dose–response analysis of compounds **17** (–■–)- and **32** (· ·■· ·) on colony-stimulating activity from C6 stromal cells. CFU-GM numbers are presented as percentage of control.

## Experimental Section

**Peptide Synthesis.** All reagents and solvents for peptide synthesis were reagent grade and used without further purification. The peptides were synthesized by solid phase techniques using either a manual shaker vessel or an Applied Bio System Model 430 A peptide synthesizer. Protected amino acid derivatives were purchased from Bachem Biosciences (PA). Peptides with a C-terminal carboxylate were synthesized using phenylacetamidomethyl resin (PAM), and peptides with a C-terminal carboxamide were synthesized using benzhydrylamine resin (BHA). The derivatized resins were purchased from Applied Biosystem Inc., CA. The α-amino groups of the amino acids were protected by *t*-Boc protecting groups, and the side chain protecting groups were as follows: L- and D-Asp, OBzl; L- and D-Glu, OBzl; L-Lys, 2-Cl-Z; L-Ser and L-Thr, Bzl; L-His, $N^{im}$-Z; and L-Arg, $N^{\omega}$-Tos.

Each amino acid, except the diamino dicarboxylic acids, was coupled sequentially to the peptide chain grown from the C-terminal amino acid using 3-fold excess of the protected amino acid derivative, 3 equiv each of *N*,*N*′-dicyclohexylcarbodiimde (DCC) and 1-hydroxybenzotriazole hydrate (HOBt) in DMF/DCM (1:1). The diamino dicarboxylic acids were coupled with 0.5 equiv of acid derivative using 1 equiv each of DCC and HOBt. If the coupling was not complete after 48 h as monitored by a Kaiser ninhydrin test,[20] an additional 1 equiv of DCC and HOBt were added and the reaction was allowed to proceed for another 24 h. The peptides were cleaved from the resin with concomitant side chain deprotection using anhydrous HF at −5 °C for 1 h.[33]

The crude peptides were extracted in aqueous 0.1% TFA and purified by preparative reverse phase HPLC (Vydac C-18 column, 2.2 × 25 cm, 5 $\mu$m particle size). An ascending linear gradient of aqueous 0.1% TFA and acetonitrile containing 0.1% TFA at a flow rate of 5 mL/min was used for elution. The fractions containing the product were pooled and lyophilized. All peptides were shown to be homogeneous by analytical reverse phase HPLC (Vydac C-18 column, 0.46 × 25 cm, 5 $\mu$m particle size) using an ascending linear gradient of aqueous 0.1% TFA and acetonitrile containing 0.1% TFA at a flow rate of 1 mL/min for elution. The peptides were also analyzed using isocratic conditions. The peptides were characterized by amino acid analysis (6 N HCl at 110 °C for 24 h, performed on a Dionex Autoion 100 analyzer) and FAB mass spectrometry (VG Zab high-resolution mass spectrometer).

**Preparation of 1.** A manual shaker vessel was charged with Boc-Lys(2-Cl-Z) resin (23 g, 0.49 mmol/g, 11.3 mmol). The resin was treated with 40% TFA/$CH_2Cl_2$ (40 mL) for 5 min followed by an additional 40% TFA/$CH_2Cl_2$ (40 mL) for 30 min. The resin was washed with $CH_2Cl_2$, and the resulting TFA salt was neutralized with 10% DIEA/$CH_2Cl_2$. The resin was further washed with $CH_2Cl_2$ and then treated with (2*S*,7*S*)-

2,7-bis(*tert*-butoxycarbonyl)diaminosuberic acid (2.29 g, 5.67 mmol), DCC (2.34 g, 11.34 mmol), and HOBt (1.53 g, 11.34 mmol) in DMF (20 mL) and $CH_2Cl_2$ (20 mL). After 76 h, the resin was extensively washed with $CH_2Cl_2$ and DMF. Five grams of this resin was used to complete the synthesis of the target peptide.

The resin was treated with TFA and neutralized with DIEA as described above. The resin was then treated with Boc-Asp(OBzl) (3.67 g, 11.34 mmol), DCC (2.34 g, 11.34 mmol), and HOBt (1.53 g, 11.34 mmol) in $CH_2Cl_2$ (20 mL) and DMF (20 mL). In a similar fashion, Boc-Glu(OBzl) (11.34 mmol) and pGlu (11.34 mmol) were coupled to the growing peptide chains. The resin was washed with 40% TFA/$CH_2Cl_2$ and dried.

One gram of the resin, prepared as above, was transferred into an HF reaction vessel. Anisole (1.75 mL) was added, and the reaction vessel was chilled to −70 °C. Anhydrous HF (20 mL) was condensed in the vessel, and the mixture was allowed to warm to −5 °C. After 1 h, the HF was evaporated, and the resulting peptide resin mixture was transferred to a sintered glass funnel.[33] The mixture was washed with ether, and the peptide was extracted into 0.1% aqueous TFA (100 mL). The solution was frozen and lyophilized to dryness. The crude peptide was dissolved in 0.1% aqueous TFA (1.0 mL), loaded onto a preparative HPLC column (Vydac, 2.2 × 25 cm, 5 $\mu$m particle size), and eluted with a linear gradient of increasing concentration of acetonitrile containing 0.1% TFA (0−20% in 60 min) at a 5 mL/min flow rate. The fractions containing product were pooled and lyophilized to dryness to give the title compound (146 mg). The purity and structure of the peptide were confirmed by analytical HPLC, FAB mass spectroscopy, and amino acid analysis.

All other peptides were synthesized in an analogous fashion.

**Preparation of Test Compounds or Biological Assays.** All test compounds were dissolved in water at 1 mg/mL concentration, and 100 $\mu$L of this solution was lyophilized in Nalgene cryovials. Thus each vial contained 100 $\mu$g of the compound. The vials were stored in a −20 °C freezer, and just before the experiment compounds were dissolved in phosphate-buffered saline (PBS) before addition to *in vitro* culture. All test solutions were free of detectable endotoxin as determined by a commercially available kit with a detection limit of 0.03 EU/mL.[31,32]

**Murine Stromal Cell Line (C6)**. C6 cell line was derived from long term culture of murine bone marrow fibroblasts. Several passages of adherent cells, twice weekly for several weeks, and then cloning by limiting dilution yielded a fibroblastic cell line that responded to SK&F 107647 by producing colony-stimulating activity. This cell line, termed as C6 as well as a subclone C6.4, was used for screening the analogues. Cells were grown to confluence in 6-well (35 mm) tissue culture dishes in RPMI medium with 10% FBS and a tissue culture grade penicillin and streptomycin mixture (GIBCO, Grand Island, NY). Upon reaching confluence, stromal cell culture medium was changed to serum free medium with or without addition of various concentrations of the test peptide (10 $\mu$L). Total incubation volume was 1 mL. Analogues were tested at multiple log doses in duplicate wells. Initial testing of analogues was at 0.001, 0.01, 0.1, 1, 10, 100, and 1000 ng/mL. Modifications in this dose range up or down occurred post-analysis of the initial experiments. Stromal cells were incubated for 1 h at 37 °C and then washed three times with warm medium; 1 mL of serum free medium was added back to the stromal cells, and after 24 h cell free supernatants were collected, sterile-filtered, and stored at 4 °C before being assayed for the presence of CSA activity.

**Colony-Forming Assay.** Specific marrow progenitor cells committed to granulocytes macrophage were quantitated as previously described.[14] Briefly, femoral bone marrow cells were obtained from C57Bl/6 mice and adjusted to $10^6$ cells in McCoy medium without serum. A single-layer agar system utilized the following: McCoy medium enriched with nutrients ($NaHCO_3$, pyruvate, amino acids, and vitamins), 0.3% Bacto agar, and 15% fetal bovine serum. To this mixture were added C6 cell line supernatant (10−2.5%, v/v) from above along with murine bone marrow cells (final concentration $10^5$ cell/mL). The agar plates were incubated at 37 °C, 7.5% $CO_2$ in humidified air for 7 days. Colonies (>50 cells) of proliferating bone marrow cells (CFU-GM) were quantitated by microscopy. The number of granulocytes macrophage colonies is proportional to the amount of colony-stimulating activity present within the C6 supernatants. Stromal cell supernatants from compound-treated cultures were analyzed in triplicate wells at each concentration tested.

**Statistical Analysis**. The mean values obtained from experimental groups were compared to controls using a two-tailed Student's *t*-test ($p < 0.05$ indicated significant difference). $EC_{50}$ values were estimated from the compound's dose-response curve. Maximum activity of every compound tested was equivalent to CSA induced by **1**.

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(26) The extreme potency of **1** complicates the interpretation of these results. These analogues may be devoid of activity since one cannot rule out the possibility that the observed (diminished) activity may result from a minor contamination of **1**.
(27) Substitution of cyclopentanecarboxylic acid, 2-furoic acid, 3-furoic acid, 5-oxo-2-tetrahydrofurancarboxylic acid, 2-oxo-4-thiazolidenecarboxylic acid, 2-tetrahydrofurancarboxylic acid, 3-tetrahydrofurancarboxylic acid, 2- thiophenecarboxylic acid, 3-thiophenecarboxylic acid, tyrosine, and pyridineacetic acid at position 1 of **1** results in analogues that are inactive at the highest dose tested (1 μg/mL).
(28) Substitution of bicyclic carboxylic acids, *e.g.*, 4-hydroxyquinoline-2-carboxylic acid, indole-2-carboxylic acid, 1-isoquinolinecarboxylic acid, 3- isoquinolinecarboxylic acid, and qunioline-2-carboxylic acid, at position 1 of **15** yielded analogues that were inactive at the highest dose (1 μg/mL) tested.
(29) Synthesis of (2*S*,8*S*)-diaminoazelaic acid and (2*S*,9*S*)-diaminosebacic acid are reported in PCT WO 93/24523.
(30) Substituiton of $Lys^5$ with Ala, Asp, Glu, 4-amino-L-phenylalanine, (2*S*)-2,4-diaminobutyric acid (Dba), and 1,6-diaminohexane (Dhe) yielded analogues that were inactive at the highest dose (1 μg/mL) tested.
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(32) The commercial kit for endotoxin testing was purchased from Associate of Cape Cod, Inc., MA.
(33) HF is a highly toxic, low-boiling (bp 19 °C) corrosive acid. A Teflon apparatus (available commercially) must be used for cleavage reactions.

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