Design of Low Molecular Weight Hematoregulatory Agents from the Structure−Activity Relationship of a Dimeric Pentapeptide

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We report herein, a new class of simple hematoregulatory semipeptides, formally derived from the cystine-dimerized peptide pGlu-Glu-Asp-Cys-Lys-OH, where the disulfide bond has been replaced by an isosteric dicarba bridge. The structure−activity relationship (SAR) of a series of analogues incorporating replacements at positions 1 and 2 of peptide **1** led to the design of active conformationally constrained cyclic peptides (**12**, **13**). Ring closure was achieved by cyclization of the *N*-terminal amino groups at position 2 of peptide **2** using pyrazine-2,3-dicarboxylic acid. Subsequent excision of the putative C-terminal scaffold domain from the active cyclic peptides resulted in the discovery of a new class of low molecular weight hematoregulatory agents exemplified by compound **16**. This semipeptide analogue, comprising two D-Ser residues connected via amide bonds to the acid groups of pyrazine-2,3-dicarboxylic acid, had comparable biological activity to the lead peptide **1**. The stereochemical requirements for the observed biological activity of these novel compounds were examined. Furthermore, the hematopoietic synergistic activity induced by compound **16** in stromal cell cultures was blocked by an antibody known to neutralize the hematoregulatory effect of **1**, indicating a common mechanistic end point. Compounds of the class typified by **16** may form the basis for the development of novel therapeutic agents within the area of immunoregulation.

Introduction

The process known as hematopoiesis, whereby stem cells proliferate and differentiate into mature blood cells, has a critical role in protection against pathogenic invasion.[1] Several proteinaceous factors termed colony-stimulating factors (CSFs), including G-CSF and GM-CSF, have already demonstrated clinical utility by enhancement of host defense mechanisms.[2]

In 1982, Paukovits isolated a thiol-containing compound from mature granulocytes with potent myelopoietic inhibitory activity.[3] The active component was presumed, following analysis and chemical synthesis, to be a pentapeptide of amino acid sequence pGlu-Glu-Asp-Cys-Lys.[4] In direct contrast to the inhibitory effect of the monomeric peptide, dimerization through disulfide formation generated a peptide with potent stimulatory activity.[5] Subsequent replacement of the disulfide bond with an isosteric ethylene spacer resulted in the novel nonreducible hematoregulatory peptide **1** (Figure 1).[6] This peptide demonstrated potent anti-infective activity in models of bacterial sepsis[7] and *Candida albicans*[8] infection, significantly increasing the survival rates of treated animals given a lethal challenge of pathogen. It has been shown to stimulate indirectly hematopoiesis[9] and enhance effector cell function by the induction of a proteolytically modified form of the chemokine KC from murine bone marrow stromal cells.[10] The truncated form of this protein, consisting of residues 5−72, was isolated from supernatants of a marine stromal cell line following stimulation with peptide **1**. Furthermore, KC[5-72] had no effect on GM-CFC colony formation alone but was a synergistic factor along with either M-CSF, G-CSF, GM-CSF, or IL-3.[11] KC[5-72] has therefore been termed hematopoietic synergistic factor (HSF).

As part of a general structure−activity relationship (SAR) study surrounding peptide **1**, a picture emerged of a pharmacophore region comprising the two dipeptide sequences pGlu[1]-Glu[2] shown in Figure 1. The amino acid residues at positions 3−5 were believed to form a salt-bridged framework with the two pharmacophore halves protruding from the scaffold-like structure. Bhatnagar et al.[12] reported that substitution at position 1 with picolinic acid and at position 2 with Ser resulted in a peptide, **2**, with significantly improved potency.

In this study we report on how the existing SAR surrounding peptide **2** was extended by additional substitutions at position 1 and how this information was used to design conformationally constrained cyclic analogues. The observed biological activity of cyclic peptides **12** and **13** (Table 2), where the pharmacophore region had considerably reduced mobility, showed that it was possible to lock the critical functional elements into a bioactive conformation. Subsequent removal of the now redundant scaffold region, known to be essential in noncyclic analogues, led to the design of the novel class of semipeptides shown in Table 3, with full HSF-inducing activity.

Chemistry

Peptides **1**−**13** were synthesized using solid-phase techniques based on the 9-fluorenylmethoxycarbonyl (Fmoc) amino protection methodology[13] employing resin linkers cleavable with trifluoroacetic acid (TFA). *N*,*N*′-Di-Fmoc-protected L,L-2,7-diaminosuberic acid (Sub) was prepared by Kolbe electrolysis of Boc-Glu-OBn using the experimental conditions described by Nutt et al.[14] Modifications to the standard assembly protocols were necessary to obtain the dimeric target structures. Acylation of the H-Lys(Boc) resin with 0.33 mole equiv of *N*,*N*′-di-Fmoc-protected L,L-2,7-diaminosuberic acid was performed followed by an additional coupling cycle

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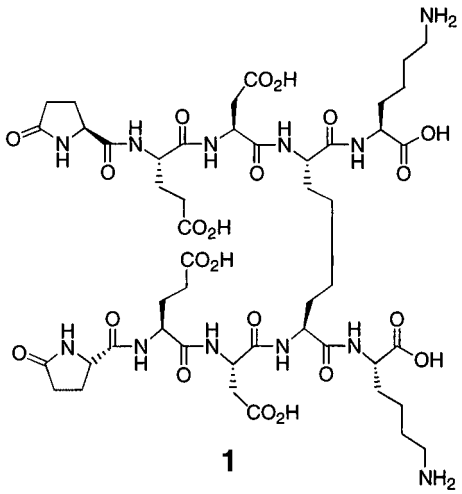
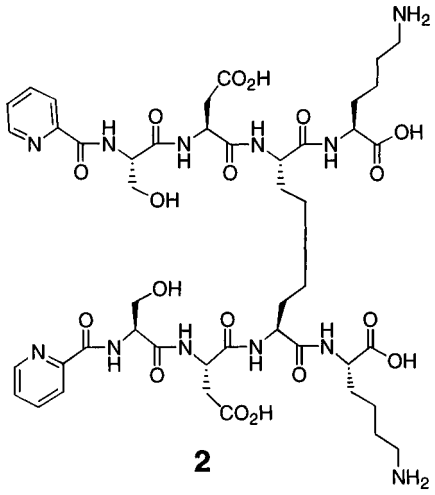


1

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



2

(Pic-----Ser----Asp)$_2$---Sub----(Lys)$_2$

Position 1 2 3 4 5

Figure 1. Structure and numbering system used for peptide **1** and its more potent analogue **2**.

with dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBt) in order to ensure anchoring of both carboxyl groups of the Sub residue to the Lys amino groups on the solid support. Excess resin-bound amino groups were then capped by acetylation prior to continuation of chain extension. On-resin N-terminal cyclization in the case of peptides **11**−**13** was accomplished as follows: [Fmoc-Ser(Bu^t)Asp(OBu^t)]$_2$-Sub-[Lys(Boc)]$_2$ peptidyl resin was Fmoc deprotected with piperidine in *N*,*N*-dimethylformamide (DMF) and reacted with 0.5 mole equiv (with respect to resin-bound amino groups) of pyrazine-2,3-dicarboxylic acid, activated with 1 mole equiv of benzotriazol-1-yloxytris-(pyrrolidino)phosphonium hexafluorophosphate (PyBOP).[15] This was followed by a second step using only coupling reagent to effect cyclization. After complete chain assembly, peptides were simultaneously side chain deprotected and cleaved from the solid support followed by precipitation of the crude material from diethyl ether. The crude peptides were purified by preparative reverse-phase HPLC.

Semipeptide analogues containing one L- and one D-Ser residue (**15**, **20**) were synthesized starting with a commercial peptide synthesis resin substituted with Fmoc-L-Ser(Bu^t). The diacid component was first coupled in large excess to the deprotected resin using 0.5 mole equiv (with respect to acid groups) of the coupling reagent *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU).[16] After washing with DMF, a second 0.5 mole equiv of HATU was added and preactivation of resin-bound acid groups allowed to proceed for 5 min before addition of an excess of H-D-Ser(Bu^t)-OBu^t. Semipeptides containing two Ser residues of the same chirality were synthesized in solution using the appropriate aromatic diacids and *tert*-butyl-protected (side chain and carboxyl group) L- or D-Ser derivatives.

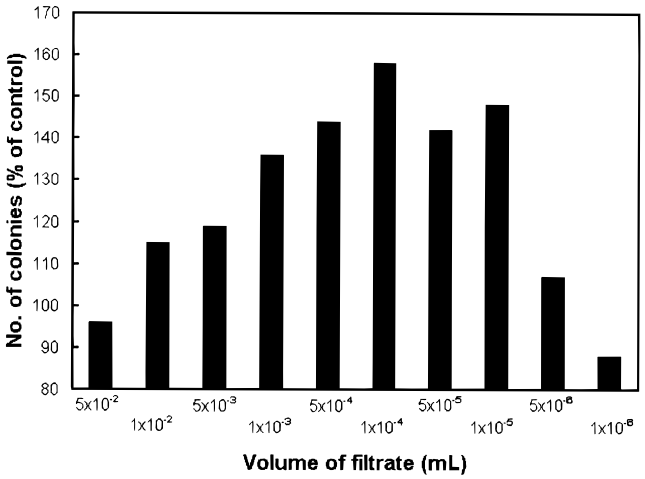


Figure 2. 'Bell-shaped' dose−response curve obtained when HSF-containing supernatants from C6.4 cells stimulated with 1 μg/mL peptide **1** were added to GM-CFC cultures in the presence of a suboptimal amount of L929 cell-conditioned medium.

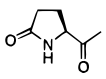
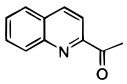
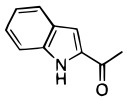
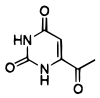
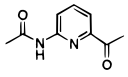
All compounds submitted for biological testing were purified to homogeneity by preparative reverse-phase HPLC. Final purity and structural integrity were assessed by analytical HPLC, quantitative amino acid analysis, NMR, fast atom bombardment mass spectrometry (FAB-MS), high-resolution mass spectrometry (HR-MS), and polarimetry, as appropriate. Diastereomeric peptides[17] and semipeptides[18] were additionally analyzed by HPLC cochromatography.

Biology

In vitro biological activity was related to the amount of HSF induced following incubation of test compound with the established bone marrow-derived stromal cell line C6.4.[12] The HSF contained in the cell supernatants, together with a suboptimal amount of CSFs from L929 cell-conditioned medium, significantly increased the number of colonies formed in a GM-CFC assay compared to CSF alone. In practice, the activity was calculated from a dose−response curve using a dilution series of conditioned medium from the peptide-stimulated cell line. The activity induced by compound **1** in this assay, shown in Figure 2, followed a bell-shaped dose−response curve as previously reported.[12] The HSF activity was calculated by dividing the increase in colony number over background by the smallest volume (mL) of supernatant giving rise to a significant increase in colonies. The values, given in HSF units/mL of supernatant, are an average of several experiments where the mean value obtained from each

Table 1. Extended SAR of Hematoregulatory Activity at Positions 1 and 2

$(R\text{-}Yaa\text{-}Asp)_2\text{-}Sub\text{-}(Lys)_2$

no.	R	Yaa	HSF units/mL[a]
1		L-Glu	1.7×10^5
3		L-Ser	0
4		L-Ser	0
5		L-Ser	1.9×10^4
6		L-Ser	1.2×10^5
7		L-Ser	2.4×10^6
8		L-Ser	2.8×10^6
9		L-Ser	0
10		L-Ser	1.0×10^6

[a] All peptides tested at a dose of 1000 ng/mL.

experimental group was compared to control using the two-tailed Student's *t*-test.

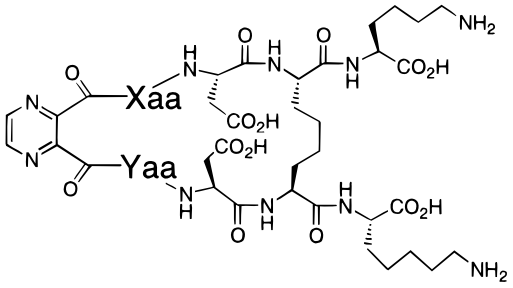
Polyclonal IgG antibodies, raised against the synthetic peptide fragment 38−72 of the chemokine KC,[19] were added to the supernatants collected from peptide-stimulated C6.4 cells immediately before addition to the GM-CFC assay. Incubation with this antibody had no effect on the background colony numbers produced in the assay by the CSFs contained in the L929 cell-conditioned medium.

Biological Results and Discussion

The aim of this work was firstly to examine more closely the SAR at position 1 of the highly potent peptide **2**. This, it was hoped, would provide further evidence for the developing pharmacophore hypothesis and ultimately permit the design of simpler, low molecular weight hematoregulatory agents.

Substitution of the picolinoyl moiety of peptide **2** with a variety of heterocyclic carboxylic acids was carried out and the activity in the HSF assay determined (Table 1). Peptides containing the position 1 substitutions orotic acid, **5**, 2-pyrazinecarboxylic acid, **6**, 6-*N*-acetylpicolinic acid, **7**, 3-hydroxypicolinic acid, **8**, and 2-pyrrolecarboxylic acid, **10**, were all found to be active, while the larger heterocyclic rings of quinaldic acid, **3**, 2-indolecarboxylic acid, **4**, and 1-isoquinolinecarboxylic acid, **9**, had no detectable activity. These results showed that a diverse range of substitutions was tolerated provided the steric bulk at position 1 was not significantly increased. Furthermore, the presence of a nitrogen atom α to the carboxyl group of position 1 was not enough to overcome the apparent loss of biological activity caused by the increase in bulk. These obervation were in agreement with the previously published data for peptide **1**[12] and may reflect the shape of the receptor binding pocket of these compounds.

Table 2. SAR of a Series of Cyclic Peptides



no.	Xaa	Yaa	HSF units/mL[a]
11	L-Ser	L-Ser	0
12	L-Ser	D-Ser	5.7×10^5
13	D-Ser	D-Ser	2.3×10^6

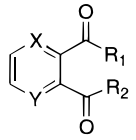
[a] Cyclic Peptides tested at a dose of 100 ng/mL.

In an attempt to design a conformationally constrained cyclic peptide, we postulated that the structural requirements described above would be maintained in the two pharmacophore halves were replaced by a bifunctional, monomeric mimetic. Knowing that the pyrazine rings of peptide **6** were tolerated, we chose pyrazine-2,3-dicarboxylic acid as a possible candidate to effect ring closure of the position 2 Ser residues via amide bond formation, as illustrated by the general structure in Table 2. In this way it was possible to retain both nitrogen atoms α to the dicarboxylic acid while satisfying conditions of size and symmetry. To our surprise peptide **11**, the all L-Ser peptide, had no detectable activity, while the peptides **12**, containing one D- and one L-Ser, and **13**, containing two D-Ser residues, had levels of activity comparable to peptide **1**.

We rationalized from these results that the scaffold region (positions 3−5) in the cyclic peptide series no longer played a significant role in determining the correct orientation at positions 1 and 2. Due to the constrained nature of the pyrazine ring system, it seemed likely that the pharmacophore mimetic alone should possess the necessary functionality and conformational integrity required for biological activity. It was anticipated that the scaffold region of the cyclic peptide had now become a redundant feature of the molecule.

Semipeptides, representing the new constrained pharmacophore (Table 3), were synthesized and tested for their ability to induce HSF. As for the dimer peptides, the activity was found to depend on the stereochemistry

Table 3. SAR for a Series of 2,3-Disubstituted Dicarboxylic Acids Containing Combinations of D- and L-Ser



no.	X	Y	R_1	R_2	HSF units/mL[a]
14	N	N	L-Ser-OH	L-Ser-OH	0
15	N	N	L-Ser-OH	D-Ser-OH	1.6×10^5
16	N	N	D-Ser-OH	D-Ser-OH	1.9×10^5
17	CH	N	L-Ser-OH	L-Ser-OH	0
18	CH	N	D-Ser-OH	D-Ser-OH	1.2×10^6
19	CH	CH	L-Ser-OH	L-Ser-OH	0
20	CH	CH	L-Ser-OH	D-Ser-OH	0
21	CH	CH	D-Ser-OH	D-Ser-OH	1.3×10^5

[a] Semipeptides tested at a dose of 100 ng/mL.

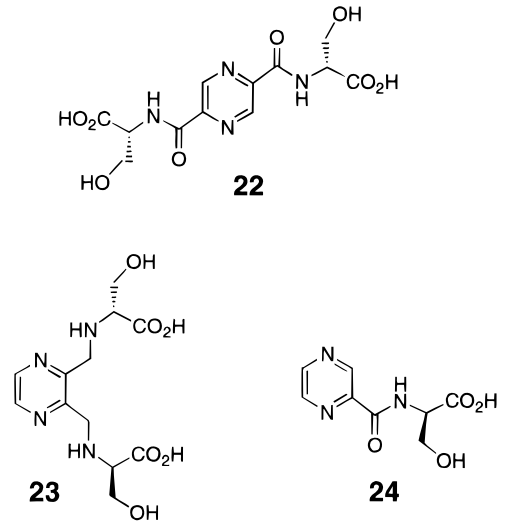


Figure 3. Inactive D-Ser analogues demonstrating the importance of both the 2,3-disubstituted pyrazine ring system and the constraining amide bonds.

of the Ser residues. The L-Ser analogues **14**, **17**, and **19** were inactive, while a comparison of compounds **15** and **20**, comprising one D- and one L-Ser residue coupled to the pyrazine and phthalic acid rings, respectively, demonstrated an activity requirement for the ring nitrogen atom. In contrast, analogues **16**, **18**, and **21**, comprising two D-Ser residues in combination with the pyrazine-2,3-carboxylic, quinolinic, and phthalic acid ring systems, respectively, retained full activity, with no obvious heteroatom dependence α to the carboxyl groups.

The compound **16** was chosen as a model structure to investigate more closely the effect on activity when other functional groups were removed or modified. The *β*-hydroxyl groups of the Ser side chains appeared to be important in the semipeptide series. Analogues containing either two D-Ala, two Gly, or one D-Ser and one Gly residue were devoid of activity. The D-Ser carboxylic acid termini in the pyrazine series were also important since the C-terminal diamide analogue of **16** was inactive (unpublished results).

The three analogues **22**−**24**, shown in Figure 3, were designed to answer other crucial questions on semipeptide SAR. Compound **22**, where two D-Ser residues were incorporated into the pyrazine-3,6-dicarboxylic acid ring, was inactive, as was the reduced amide bond analogue **23**. This demonstrated a requirement for the 2,3-disubstituted ring system, as well as the presence of the constraining amide bonds between the ring and the D-Ser residues. Finally, analogue **24**, derived from pyrazine-2-carboxylic acid and D-Ser, showed that two Ser residues were required for activity.

Table 4. Neutralization of Colony-Stimulating Activity from Active Supernatants on Incubation with Polyclonal IgG Antibodies Raised against KC[38-72]

no.	active supernatant, HSF units/mL	active supernatant + antibody,[b] HSF units/mL
1[a]	1.7×10^5	0
12	5.7×10^5	0
13	2.3×10^6	0
15	1.6×10^5	0
16	1.9×10^5	0

[a] Test dose 1000 ng/mL. Others tested at 100 ng/mL. [b] Antibody used at 1:10000 dilution.

The level of *in vitro* activity for each of the test compounds was expressed in HSF units/mL. This measure of activity relates directly to the amount of HSF produced by the C6.4 cells on stimulation with a given dose of test compound. For the dimer analogues listed in Table 1, a dose of 1000 ng of peptide/mL of stromal cell culture medium was found to be optimal, giving the typical bell-shaped curve shown in Figure 2. The active cyclic peptides and semipeptide analogues gave a similar curve (data not shown) but at an optimal dose of 100 ng of compound/mL of stromal cell culture medium. The more rigid analogues therefore possessed a specific activity 10 times greater than their more flexible dimeric precursors. Furthermore, incubation of supernatants from stromal cells stimulated with compounds **1**, **12**, **13**, **15**, and **16** with an antibody raised against KC[38-72][19] completely blocked synergistic activity, implicating this chemokine product in the mechanism of both peptide and semipeptide analogues (Table 4).

We have shown therefore in this SAR study that it was possible to design, in a rational manner, active hematoregulatory agents of much reduced complexity to peptide **1**. The simplicity of this new class of hematoregulatory semipeptides may preclude the discovery of bioavailable analogues of increased utility. Although the receptors have not yet been identified, a broadening knowledge of the downstream molecular events triggered by these compounds may pave the way for the development of new therapeutic agents.

Conclusion

We have further extended the SAR data of peptide **2**, incorporating a range of substituents at position 1. From a series of tolerated substitutions, the pyrazine ring system was chosen as a suitable template for the synthesis of conformationally constrained cyclic peptides. The fully cyclic analogues, **11**−**13**, were prepared using pyrazine-2,3-dicarboxylic acid as a monomeric position 1 pharmacophore mimetic. The activity of cyclic peptides **12** and **13** revealed a stereochemical requirement for at least one D-Ser residue at position 2. Furthermore, excision of the conformationally constrained pharmacophore mimetic from the putative scaffold region led to a series of active semipeptides exhibiting the same stereochemical requirement as the fully cyclic structures.

The results reported here provide further evidence for a model consisting of a scaffold region in peptide **1**

furnished by positions 3−5, which allow for the correct orientation of the position 1 and 2 pharmacophore halves. This model has been used with success in the development of analogues of considerably reduced chemical complexity. The low molecular weight of the active semipeptides described herein may lead to the development of new classes of readily synthesizable and potentially bioavailable immunoregulators.

Experimental Section

Peptide Synthesis. All reagents and solvents for peptide synthesis were of reagent grade and were used without further purification. The peptides were synthesized by solid-phase techniques using a manual nitrogen bubbler apparatus.[20] All amino acid derivatives were purchased from Novabiochem and heterocyclic acid derivatives used as supplied by Fluka. Peptides were synthesized using 2-methoxy-4-alkoxybenzyl alcohol resin (SASRIN) preloaded with Fmoc-Lys(Boc) as supplied by Bachem. Amino acid couplings, except for diaminosuberic acid, were carried out in a stepwise manner using a 10-fold excess of protected amino acid preactivated in DMF with 1 equiv of PyBOP, 1 equiv of HOBt, and 2 equiv *N*-methylmorpholine (NMM), unless otherwise stated. Fmoc-L-Glu(OBu^t)-OH, Fmoc-L-Asp(OBu^t)-OH, and Fmoc-L-Ser(Bu^t)-OH were used for stepwise solid-phase chain assembly, while the H-L- or H-D-Ser(Bu^t)-OBu^t·HCl derivatives were used for solution synthesis.

Chromatography. Crude peptides **1**−**13** were dissolved in H_2O containing 0.1% TFA, loaded onto a preparative HPLC column (Vydac 218TP1022, 2.2 × 25 cm), and eluted at a flow rate of 5 mL/min using linear gradients of increasing MeCN concentrations in H_2O (constant concentration of TFA at 0.1%). Fractions were collected and monitored by analytical HPLC (Vydac 218TP54, 0.46 × 25 cm column; 1 mL/min, gradient elution typically from 0% to 30% MeCN in H_2O, 0.1% TFA over 20 min, $\lambda = 215$ nm). Those fractions containing target peptide with purity > 98% were pooled and lyophilized. Final yields, after chromatography, ranging from 7% to 15% pure peptide wee typically obtained.

Semipeptides **14**−**24** were purified similarly using Vydac 201HS1022 (2.2 × 25 cm) and Vydac 201HS54 (0.46 × 25 cm) columns for preparative and analytical work, respectively. The columns were developed by isocratic elution with H_2O containing 0.1% TFA. Those fractions containing target semipeptide with purity > 98% were pooled and lyophilized. Final yields, after chromatography, ranging from 60% to 70% pure semipeptide were typically obtained.

General Procedure for the Preparation of [Fmoc-Asp(OBu^t)]$_2$-Sub-[Lys(Boc)]$_2$ Peptidyl Resin. Fmoc-Lys(Boc)-SASRIN resin (5 g, 0.6 mmol/g), preswollen in DMF (5 mL), was treated with 20% piperidine in DMF for 15 min to remove the Fmoc group. After repeated DMF washing, Fmoc$_2$-Sub-(OH)$_2$ (648 mg, 1 mmol) was coupled to the deprotected resin using DCC (1.03 g, 5 mmol) and HOBt (765 mg, 5 mmol) in DMF (10 mL) for 10 h. After repeated DMF washing, a further coupling cycle with similar quantities of DCC and HOBt in DMF was performed for 5 h. Unreacted amino groups were then capped by reaction with 10% (v/v) Ac_2O/DMF (25 mL) for 15 min. Following a further Fmoc removal cycle, acylation with the pentafluorophenol ester of Fmoc-Asp(OBu^t) (5.78 g, 10 mmol) and HOBt (1.53 g, 10 mmol) was carried out in DMF (10 mL) for 2 h followed by extensive washing with DMF, CH_2Cl_2, and Et_2O. After drying *in vacuo*, the peptidyl resin was found to contain 0.36 mmol/g amino groups according to spectrophotometric measurement of Fmoc release from a weighed aliquot of resin.[21]

Preparation of Peptides 1−10. These were obtained by a general method, exemplified by the description of peptide **6**: The above [Fmoc-Asp(OBu^t)$_2$-Sub-[Lys(Boc)]$_2$ peptidyl resin (0.27 g, 0.1 mmol) was treated with 20% piperidine in DMF for 15 min to remove Fmoc groups. After repeated DMF washing, Fmoc-L-Ser(Bu^t)-OH (0.38 g, 1 mmol), preactivated with PyBOP (0.52 g, 1 mmol), HOBt (0.13 g, 1 mmol), and NMM (0.2 mL, 2 mmol) in DMF (10 mL), was coupled to the deprotected resin for 2 h. Following extensive washing with DMF, a further Fmoc deprotection cycle was carried out. Coupling of 1 mmol of pyrazine-2-carboxylic acid, preactivated with PyBOP (0.52 g, 1 mmol), HOBt (0.13 g, 1 mmol), and NMM (0.2 mL, 2 mmol) in DMF (10 mL), was then performed. The coupling was monitored using the Kaiser ninhydrin test[22] and found to be complete after 3 h. The peptide resin was then extensively washed with DMF, CH_2Cl_2, and Et_2O before drying in a stream of nitrogen. Simultaneous deprotection and cleavage of the peptide from the solid support was carried out using 5% aqueous TFA. After a reaction time of 2 h the filtered mixture was evaporated *in vacuo* and the crude peptide precipitated and washed with Et_2O before air-drying. Following purification by preparative reverse-phase HPLC (5−20% MeCN gradient over 120 min) and lyophilization, pure peptide **6** (19 mg, 10%) was obtained.

Procedure for the Synthesis of Cyclic Peptides 11 and 13. These peptides were obtained by a general method exemplified by the description of peptide **11**: [Fmoc-Asp(OBu^t)]$_2$-Sub-[Lys(Boc)]$_2$ peptidyl resin (0.55 g, 0.2 mmol) was Fmoc deprotected and washed. Fmoc-L-Ser(Bu^t)-OH (0.38 g, 1 mmol), preactivated with PyBOP (0.52 g, 1 mmol), HOBt (0.13 g, 1 mmol), and NMM (0.2 mL, 2 mmol) in DMF (10 mL), was then coupled for 2 h. After renewed Fmoc deprotection and washing, pyrazine-2,3-dicarboxylic acid (17 mg, 0.1 mmol), preactivated with PyBOP (52 mg, 0.1 mmol), HOBt (13 mg, 0.1 mmol), and NMM (20 μL, 0.2 mmol) in DMF (10 mL), was coupled for 10 h and then washed well with DMF. A second portion of PyBOP (52 mg, 0.1 mmol), HOBt (13 mg, 0.1 mmol), and NMM (20 μL, 0.2 mmol) in DMF (8 mL) was then added to the resin, and coupling continued for a further 5 h. After extensive washing with DMF, CH_2Cl_2, and Et_2O, the air-dried peptidyl resin was cleaved from the solid support in 5% aqueous TFA. After 2 h the TFA solution was evaporated *in vacuo* and the crude peptide precipitated and washed with Et_2O before air-drying. The crude peptide was purified by preparative HPLC (0−15% MeCN gradient over 40 min, 9 mL/min) and lyophilized yielding 7.2 mg (7.8%) of pure peptide **11**.

Procedure for the Synthesis of Cyclic Peptide 12. [Fmoc-Asp(OBu^t)]$_2$-Sub-[Lys(Boc)]$_2$ peptidyl resin (0.55 g, 0.2 mmol) was Fmoc deprotected, washed, and reacted with compound **15** (34 mg, 0.1 mmol), preactivated with PyBOP (52 mg, 0.1 mmol), HOBt (13 mg, 0.1 mmol), and NMM (20 μL, 0.2 mmol) in DMF (8 mL). After a reaction time of 10 h, a second portion of PyBOP (52 mg, 0.1 mmol), HOBt (13 mg, 0.1 mmol), and NMM (20 μL, 0.2 mmol) in DMF (8 mL) was added to the resin, and coupling continued for a further 3 h. Cleavage and purification procedures were identical to those outlined above. Final yield after chromatography of pure peptide **12** was 11 mg (11%).

Procedure for the Synthesis of Semipeptides 14, 16−19, 21, 22, and 24. These were obtained by a general method exemplified by the synthesis of **16**: Pyrazine-2,3-dicarboxylic acid (1 g, 6 mmol) was activated with diisopropylcarbodiimide (DIC) (0.93 mL, 6 mmol) in DMF (15 mL) for 10 min to form the anhydride *in situ*. To this was added a solution of H-D-Ser(Bu^t)-OBu^t·HCl (1.7 g, 6.6 mmol) and NMM (0.69 mL, 6.6 mmol) in DMF (10 mL). The mixture was stirred for 4 h. A second portion of DIC (0.93 mL, 6 mmol), along with HOBt (0.81 g, 6 mmol), was then added followed, after 10 min, by H-D-Ser(Bu^t)-OBu^t·HCl (1.7 g, 6.6 mmol) and NMM (0.69 mL, 6.6 mmol) in DMF (10 mL). The mixture was stirred overnight. Solvent was removed *in vacuo* and the residue redissolved in CH_2Cl_2 and extracted several times with 5% aqueous $NaHCO_3$ and water. The organic layer was dried over $MgSO_4$, filtered, and evaporated *in vacuo*. The resulting oil was chromatographed on silica gel (70:25:5 hexane/EtOAc/AcOH), and the fractions containing pure product were pooled and evaporated. The residue was then treated for 30 min with 5% aqueous TFA, evaporated *in vacuo*, redissolved in water, and lyophilized to give 1.6 g (76%) of title compound **16**. Aliquots of bulk material were further purified by preparative HPLC as described above.

Procedure for the Synthesis of Semipeptides 15 and 20. These were obtained by a general method exemplified by the synthesis of compound **15**: Fmoc-L-Ser(Bu^t)-Wang resin (1 g, 0.5 mmol/g; from Novabiochem) was Fmoc deprotected as previously described. After repeated DMF washing, pyrazine-2,3-dicarboxylic acid (0.35 g, 2 mmol), preactivated with HATU (0.76 g, 2 mmol) and NMM (412 μL, 4 mmol) in DMF (10 mL), was coupled for 2 h to the deprotected resin followed by washing with DMF (10 mL). On-resin preactivation of acid groups with HATU (0.76 g, 2 mmol) and NMM (412 μL, 4 mmol) in DMF (15 mL) was performed for 5 min before the addition of H-D-Ser(Bu^t)-OBu^t·HCl (0.5 g, 2 mmol) and NMM (0.2 mL, 2 mmol) in DMF (5 mL). Coupling was continued for a further 5 h before washing with DMF, CH_2Cl_2, and Et_2O. After the resin was dried in a stream of nitrogen, simultaneous deprotection and cleavage of the crude semipeptide from the resin was carried out in 5% aqueous TFA for 2 h. The solution was evaporated *in vacuo* and the crude semipeptide precipitated and washed with Et_2O. Aliquots of bulk material were purified by preparative HPLC as described above.

Procedure for the Synthesis of Reduced Amide Bond Semipeptide 23. 2,3-Dimethylpyrazine (1.08 g, 10 mmol), *N*-chlorosuccinimide (3.3 g, 25 mmol), dry benzoyl peroxide (100 mg, 0.4 mmol), and CCl_4 (100 mL) were refluxed together until no more starting material remained (48 h) as shown by thin layer chromatography (SiO_2; 5:1 hexane/Et_2O). The reaction mixture was cooled, filtered through a pad of Celite, evaporated to dryness, and then purified by silica gel column chromatography using CH_2Cl_2 as eluent to afford 1.2 g (68%) of 2,3-bis(chloromethyl)pyrazine. An aliquot of this material (0.1 g, 0.57 mmol) was redissolved in CH_2Cl_2 (10 mL) and added dropwise over several minutes to a stirred solution of H-D-Ser(Bu^t)-OBu^t·HCl (0.58 g, 2.3 mmol) and NMM (0.35 mL, 3.4 mmol). The mixture was stirred for a further 48 h and then diluted to 30 mL with CH_2Cl_2. The organic layer was extracted several times with 5% aqueous citric acid, H_2O, 5% aqueous $NaHCO_3$, and H_2O, then dried over $MgSO_4$, filtered, and evaporated *in vacuo*. The residue was treated for 30 min with 5% aqueous TFA, evaporated *in vacuo*, redissolved in water, and lyophilized. Further purification of an aliquot of crude material by preparative HPLC was carried out as described above yielding 20 mg (44%) of peptide **23**.

Murine Stromal Cell Line (C6.4). A murine fibroblast-like cell line was obtained from A. King (SmithKline Beecham Pharmaceuticals, PA). The parental cell line, C6, was first derived from the adherent cell layer of a long-term bone marrow culture. Several biweekly passages of the adherent cells and cloning by limited dilution yielded a cell line that responded to peptide **1** by releasing a factor that synergized with a colony-stimulating factor in a GM-CFC assay. Later this cell line was subcloned by limiting dilution and a new clone, C6.4, obtained which was used throughout this study for the screening of peptide analogues.

Preparation of Test Compounds for Biological Assays. All test compounds were dissolved in water containing 0.1% TFA at a concentration of 1 mg/mL and dispensed into Nalgene cryovials before lyophilization. Each vial, containing 0.1 mg of compound, was stored at −20 °C and diluted immediately prior to an experiment in 1 mL of phosphate-buffered saline (PBS) containing 1% fetal bovine serum (FBS) (Hyclone Laboratories, Utah), 100 U/mL penicillin, and 100 μg/μL streptomycin (Bio Whittaker, Walkerville, MD) before addition to *in vitro* cultures.

HSF Induction. C6.4 cells were grown to confluence in 12-well tissue culture dishes (Costar) in RPMI 1640 medium with 15% FBS, 100 U/mL penicillin, and 100 μg/μL streptomycin before the addition of either PBS or test compound solution. Analogues were tested at multiple doses in duplicate wells; those doses yielding optimal results were selected for further testing. Dimer peptides (**1**−**10**) were typically tested at 1000 ng/mL, while cyclic and semipeptide analogues (**11**−**24**) were tested at 100 ng/mL in a total incubation volume of 1 mL. The cultures were incubated for 18 h at 37 °C with 5% CO_2 in air (Nuair incubator). The supernatants were harvested and filtered through a Centricon 30 kD a cutoff membrane (Amicon, Beverly, MA).

Antibodies to HSF. Polyclonal IgG antibodies were raised in goats against a fragment of synthetic KC polypeptide corresponding to the sequence 38−72.[19] The IgG fraction was purified using protein G/Superose-12 (Pharmacia) and was found to be more than 98% pure. The antibody preparation (3.8 mg/mL) was used at 1:10000 dilution, which completely blocked the activity of recombinant KC[5-72] as well as the HSF activity induced by 1 μg/mL compound **1**. The antibody had no effect on the background colony numbers produced by the suboptimal amounts of L929 cell-conditioned medium used in the GM-CFC assay.

Granulocyte/Macrophage Colony-Forming Cell (GM-CFC) Assay. Multiple dilutions of filtrates were added to 75 000 femoral bone marrow cells obtained from C57bl/6 mice (Bomholt Gaard, Denmark) suspended in McCoys medium (Gibco, Paisley, Scotland) enriched with nutrients ($NaHCO_3$, pyruvate, amino acids, and vitamins) and containing 100 U/mL penicillin, 100 μg/mL streptomycin, and 15% FBS. To this was added a predetermined suboptimal amount of L929 cell-conditioned medium, known to produce between 20% and 30% of maximum colony number, along with 100 μL of 3.3% agar (Difco Laboratories, MI) to make a final volume of 1 mL. Cultures were incubated for 7 days in an incubator (Nuair) at 37 °C and 5% CO_2 in air. Colonies were inspected using an inverted microscope (Zeiss, Germany), and those containing more than 50 cells were scored.

Statistical Analysis. The synergistic activity was determined from the most dilute sample (i.e., smallest volume of added filtrate in milliliters) giving rise to a significant increase in colony numbers over background. By defining 1 unit of HSF as the activity generating one colony above background, the following formula was used to calculate the units of HSF/mL of supernatant: HSF units/mL = (colonies in test group− colonies in background)/amount of filtrate added. The mean value obtained from each experimental group was compared to control using the two-tailed Student's *t*-test.

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(17) Peptide **13** was analyzed by coinjection with peptide **12** on analytical HPLC (Vydac 218TP54, 0.46 × 25 cm column). Following elution with a linear gradient of increasing 0.1% TFA/MeCN (0−30% over 20 min) at a flow rate of 1 mL/min, two peaks were observed corresponding to compound **13** at 11.3 min and compound **12** at 11.7 min.

(18) Semipeptides **15** and **20** containing a mixture of D- and L-Ser were analyzed by coinjection on HPLC with all the L isomers **14** and **19**, respectively. Two closely eluting peaks were observed. Further analysis by polarimetry in DMF of both **15** and **20** gave $[\alpha]_D = 0$, $c = 1$, for both compounds as expected. A comparison by polarimetry of the L,L and D,D isomers was also made; compound **14**, $[\alpha]_D = +17$, $c = 1$; compound **16**, $[\alpha]_D = -17$, $c = 1$; compound **19**, $[\alpha]_D = +9.1$, $c = 0.31$; compound **21** had $[\alpha]_D = -9.2$, $c = 0.32$.

(19) The C6.4 cell line and anti-KC antibody were supplied by A. King, SmithKline Beecham Pharmaceuticals, King of Prussia, PA.

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