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THE SYNTHESIS OF A HEMATOREGULATORY AGENT BASED ON HP-5B CONTAINING AN EFFECTIVE, ACHIRAL CYSTINE REPLACEMENT

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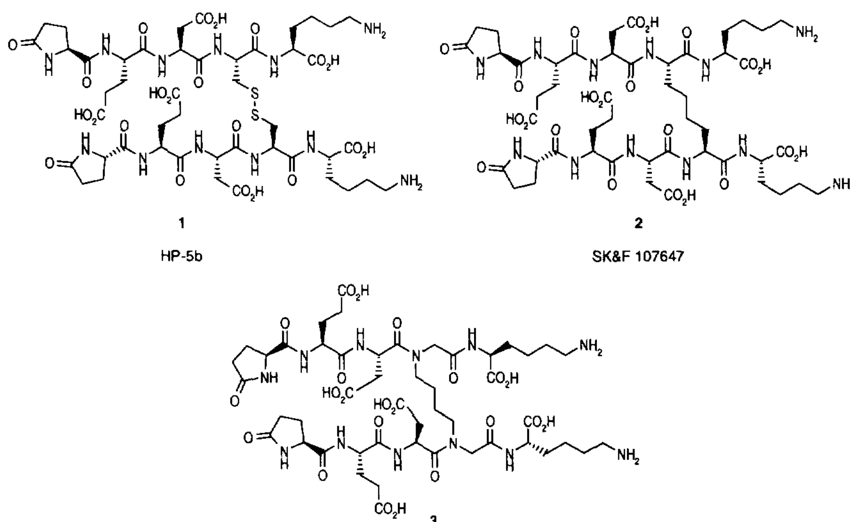
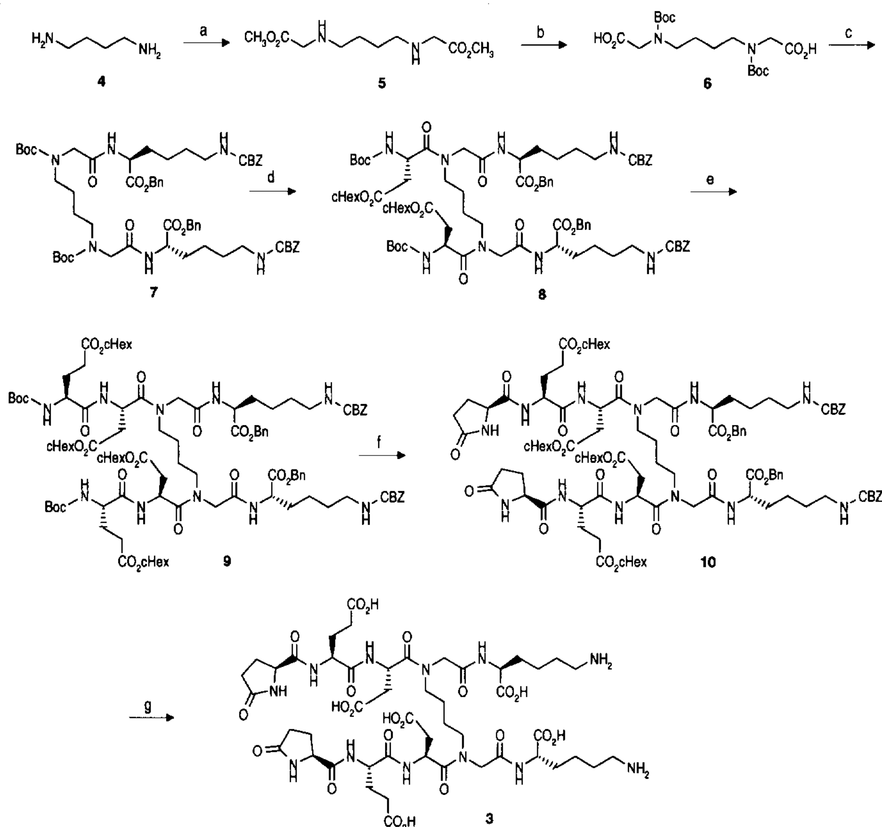
Abstract. The cystine moiety of the novel hematoregulatory peptide HP-5b has been substituted with an achiral N,N'-bis(carboxymethyl)-1,4-diaminobutane unit. The resulting compound was also a potent hematoregulatory agent, suggesting that N,N'-bis(carboxymethyl)-1,4-diaminobutane can function as an effective achiral cystine replacement in this molecular series. Copyright © 1996 Elsevier Science Ltd

Hematopoiesis is the means by which pluripotent stem cells repopulate the various unipotential blood cell lines through a hierarchical process of proliferation and differentiation. This process is mediated at various stages by several hematopoietic growth factors and chemokines.[1] Many of these regulating factors are used clinically and others are currently in clinical trials for the treatment of myelosuppressive disorders.[2] In 1988, Laerum et al.[3] reported the discovery of hematostimulatory peptide **1**, termed HP-5b. In 1992, Veiby et al.[4] reported that this peptide indirectly enhances colony formation in murine CFU-GM in vitro by inducing a hematopoietic synergistic factor (HSF), which was not identified at the time.[5] Our initial studies on **1** yielded erratic results, which we attributed to the lability of the disulfide bond under physiological conditions.[6] We succeeded in stabilizing the molecule by replacing the bridging cystine moiety with (2*S*,7*S*)-2,7-diaminosuberic acid.[7] This compound, SK&F 107647 (**2**) retained the hematopoietic properties of **1** and was demonstrated to be effective in several models of infectious disease.[8] We have also recently isolated and characterized the HSF released from bone marrow stromal cells stimulated with **2**.[9]



Based on our structure-activity analysis of **2**, we developed a structural hypothesis in which the pyroglutamyl and glutamyl moieties function as the molecule's primary pharmacophore while the remainder of the molecule serves as part of a structural scaffold.[10] The aspartyl and lysyl moieties tolerate only limited modification and are thought to form salt bridges which help to enforce an active conformation. We wished to probe the effects of altering the diaminosuberic acid bridge, which is believed to impose activity by virtue of a combination of both spacer distance and conformational presentation of the two peptide chains. We designed **3**, in which the tetramethylene spacer of the diaminosuberic acid portion was shifted to span the nitrogens of two glycine moieties.[11] This modification generated a compound that was essentially equipotent to **2** in a colony formation assay while eliminating two chiral centers and the attendant amide protons at that amino acid position.

Scheme 1



(a) Ethylbromoacetate, Et_3N, CH_2Cl_2 (22%); (b) i. $(Boc)_2O$, Et_3N, CH_2Cl_2 (88%); ii. 1 N NaOH, THF (90%); (c) Lys(CBZ)-OBn•HCl, $i$$Pr_2NEt$, HOBt, EDC, DMF (75%); (d) i. TFA, CH_2Cl_2; ii. Boc-Asp(OcHex), $i$$Pr_2NEt$, HOBt, EDC, DMF (51%); (e) i. TFA, CH_2Cl_2; ii. Boc-Glu(OcHex), $i$$Pr_2NEt$, HOBt, EDC, DMF (30%); (f) i. TFA, CH_2Cl_2; ii. pGlu, $i$$Pr_2NEt$, HOBt, EDC, DMF (52%); (g) HF, p-cresol, 0 °C (47%).

Chemistry

Scheme 1 outlines the synthesis of analog **3**. Alkylation of **4** with ethyl bromoacetate gave the modified bridge **5** in low but acceptable yields. Major by-products of this reaction included tri- and tetra-substituted alkylation products which were easily removed by flash chromatography. Protection with di-*t*-butyldicarbonate, followed by hydrolysis of the esters furnished **6** in a protected form suitable for solution phase peptide synthesis. Coupling **6** to Lys(CBZ)-OBn by carbodiimide activation after treatment with TFA gave a satisfactory yield of **7**. Acylation of the liberated secondary amines of **7** after treatment with TFA proved to be somewhat problematic. Attempts to couple the N-terminal tripeptide, pGlu-Glu(OBn)-Asp(OBn)-CO_2H via fragment condensation with EDC/HOBt failed to produce compound **10**. Using a step-wise coupling strategy, however, compound **7** was elaborated to **10** without difficulty as shown. Treatment of **10** with anhydrous HF, followed by reverse-phase HPLC purification, afforded **3**.

Biological Results and Discussion

Compounds **1**, **2** and **3** were evaluated for the production of HSF activity from C6.4 stromal cells using a murine CFU-GM assay.[12] These compounds were analyzed in the same experiment to control for biological variation and to obtain rank order potency. Stimulation with either compounds **2** or **3** resulted in the production of approximately two orders of magnitude more HSF activity than did compound **1** (Table 1). Compounds **2** and **3** produced equivalent HSF activity within experimental error.

Table 1. HSF Activity.

Compound	HSF Activity x 10^2 (Units/mL)[13]
1	7 ± 1.4
2	143 ± 20
3	217 ± 58

The results show that replacing the (2*S*, 7*S*)-2,7-diaminosuberic acid moiety of **2** with an N.N'-bis(carboxymethyl)-1,4-diaminobutane unit (**3**) has a negligible effect on the biological activity of these compounds while simplifying the structure of **2** by eliminating two chiral centers. The enhanced potency of **3** over **1** may be attributed to either or both of the following rationales. Replacing the cystine disulfide of **1** with the ethylene spacer in **3** may yield a compound that, like **2**, is inherently more potent because of conformational and/or electronic biases. Alternatively, the enhanced potency of **3** may be due to the inherent lability of the disulfide bond of **1** under the cellular assay conditions. Varying degrees of disulfide bond reduction in **1** in vitro would decrease the amount available for HSF induction, effectively making **1** a less potent compound.

In conclusion, we have synthesized a novel hematostimulatory agent **3** based on HP-5b (**1**) and our previously reported compound SK&F 107647 (**2**). The elimination of two chiral centers with retention of equivalent biological potency represent significant advantages of the novel agent **3** over **2**. In addition, we have demonstrated that in this system, the (2*S*, 7*S*)-2,7-diaminosuberic acid and the N,N'-bis(carboxymethyl)-1,4-diaminobutane moieties can function as stable and effective cystine replacements.

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5. Abbreviations used in this manuscript: Boc, *tert*-butyloxycarbonyl; Boc-Asp(OcHex), *N*-α-*tert*-butyloxycarbonyl-aspartic acid, β-cyclohexyl ester; Boc-Asp(OBn), *N*-α-*tert*-butyloxycarbonyl-aspartic acid, β-benzyl ester; Boc-Glu(OcHex), *N*-α-*tert*-butyloxycarbonyl-glutamic acid, γ-cyclohexyl ester; Boc-Glu(OBn), *N*-α-*tert*-butyloxycarbonyl- glutamic acid, γ-benzyl ester; $(Boc)_2O$, di(*tert*-butoxy)carbonate; BOP, benzotriazol-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate; CBZ, benzyloxycarbonyl; CFU-GM, colony forming units, granulocyte/macrophage lineage; EDC, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide; HOBt, 1-hydroxybenzotriazole hydrate; HSF, hematopoietic synergistic factor; Lys(CBZ)-OBn, ε-*N*-benzyloxycarbonyl-Lysine-α-benzyl ester; pGlu, pyroglutamic acid; TFA, trifluoroacetic acid.
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12. One μg/mL of each test compound was used to stimulate cultures of C6.4 stromal cells for 24 h. Cell-free supernatants were filtered through Centricon 30,000 MW cut-off membranes (Amicon, Beverly, MA) and added to cultures containing 0.3% agar, 25 units of recombinant M-CSF and 7.5×10^4 murine marrow cells. CFU-GM were enumerated after 7 days incubation in a humidified atmosphere of 7.5% CO_2 in air.
13. HSF activity data are presented as the production of HSF units/mL ± standard error of the mean (SEM) of three replicate points. Units of HSF as a functional definition are calculated from the formula:

$$\frac{CFU_{test} - CFU_{M\text{-}CSF\ background}}{\text{mL of test supernatant}}$$

HSF values represent the lowest dilution of stromal cell supernatant which statistically increase the growth over M-CSF (25 units) stimulated CFU-GM.

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