Controlled Peptide Folding | Hot Paper

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Total Synthesis of Human Hepcidin through Regioselective Disulfide-Bond Formation by using the Safety-Catch Cysteine Protecting Group 4,4′-Dimethylsulfinylbenzhydryl**

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Abstract: *A safety-catch cysteine protecting group, S-4,4′-dimethylsulfinylbenzhydryl (Msbh), was designed and developed to expand the capabilities of synthetic strategies for the regioselective formation of disulfide bonds in cysteine-rich peptides. The directed regioselective synthesis of human hepcidin, which contains four disulfide bonds, was undertaken and led to a high-resolution NMR structure under more physiologically relevant conditions than previously. Conversely, hepcidin synthesized with the formerly assigned vicinal disulfide-bond connectivity displayed significant conformational heterogeneity under similar conditions. The two synthetic forms of human hepcidin induced ferroportin internalization with apparent EC_{50} values of 2.0 (native fold, **1**) and 4.4 nM (non-native fold, **2**), with **2** undergoing isomerization to **1** in the presence of ferroportin expressing cells.*

Hepcidin is a small peptide hormone that contains 25 amino acids and four disulfide bonds. It is involved in the regulation of systemic iron homeostasis and in the pathogenesis of several iron disorders[1] and acts by inducing the internalization of the iron exporter ferroportin in vertebrates.[1] Determination of the native connectivity of the four disulfide bonds has proved to be a daunting task with early NMR characterization suggesting a rare vicinal disulfide bond to be present; this was recently revised and the native structure, which has a different disulfide-bond connectivity, was obtained, albeit under nonphysiological conditions.[2] With the increasing impact of genomics on the discovery of novel disulfide-rich peptides, the assignment of their disulfide-bond connectivity may be solely based on 2D NMR and consequently is often plagued with ambiguity owing to the tight packing of their cysteine frameworks.[3] In principle, unambiguous regioselective[4] chemical synthesis of each potential regioisomer[5] that conforms to the NOE restraints obtained by NMR would allow the connectivity to be confidently imposed when the final structure is calculated.

The directed synthesis of up to three disulfide bonds in a cysteine-rich peptide has been accomplished by using various protecting-group schemes with either Boc or Fmoc chemistry; most commonly with combinations of *S*-triphenylmethyl (Trt), *S*-acetamidomethyl (Acm), *S*-4-methylbenzyl (Meb), *S*-4-methoxybenzyl (Mob), *S*-*tert*butyl (*t*Bu), or *S*-*tert*butylthio (S*t*Bu) groups (Boc = *tert*-butoxycarbonyl, Fmoc = 9-fluorenylmethoxycarbonyl).[4a,6] Whereas the robustness of the Trt, Acm, and Meb or Mob protecting groups is established, the removal of *t*Bu groups often results in the formation of side products and in low yields,[4a,7] and the removal of S*t*Bu groups by reducing agents has been observed to be sequence dependent.[6b,8] The only reported regioselective synthesis of a tetracystine peptide was also reliant on the use of the *t*Bu group.[9] In light of these shortfalls, we sought to expand the scope of regioselective strategies through the development of a novel safety-catch thiol protecting group and to employ it strategically to synthesize the two reported forms[2] of human hepcidin regioselectively.

Safety-catch protecting groups are designed to be stable under a desired set of conditions until made labile through specific derivatization.[10] Alkylsulfinylbenzyl-type safety-catch protecting groups have been studied in detail for the majority of natural amino acid functional groups and employed successfully in peptide synthesis.[11] In their oxidized forms, they contain electron-withdrawing sulfoxide groups and show high acid stability to trifluoroacetic acid (TFA) and hydrogen fluoride (HF) while their reduced forms, which contain electron-donating sulfide groups, are readily removed by TFA.[11a] The 4,4′-dimethylsulfinylbenzhydryl (Msbh) structure was selected for cysteine protection based on its anticipated stability during peptide synthesis and applicability for a one-step reductive acidolytic cleavage with concomitant disulfide-bond formation in TFA. We employed N^{α}-Fmoc-*S*-Msbh cysteine for the regioselective synthesis of two forms of hepcidin, specifically the native form according to Jordan et al.[2b] (**1**) and the vicinal form originally proposed by Hunter et al[2a] (**2**).

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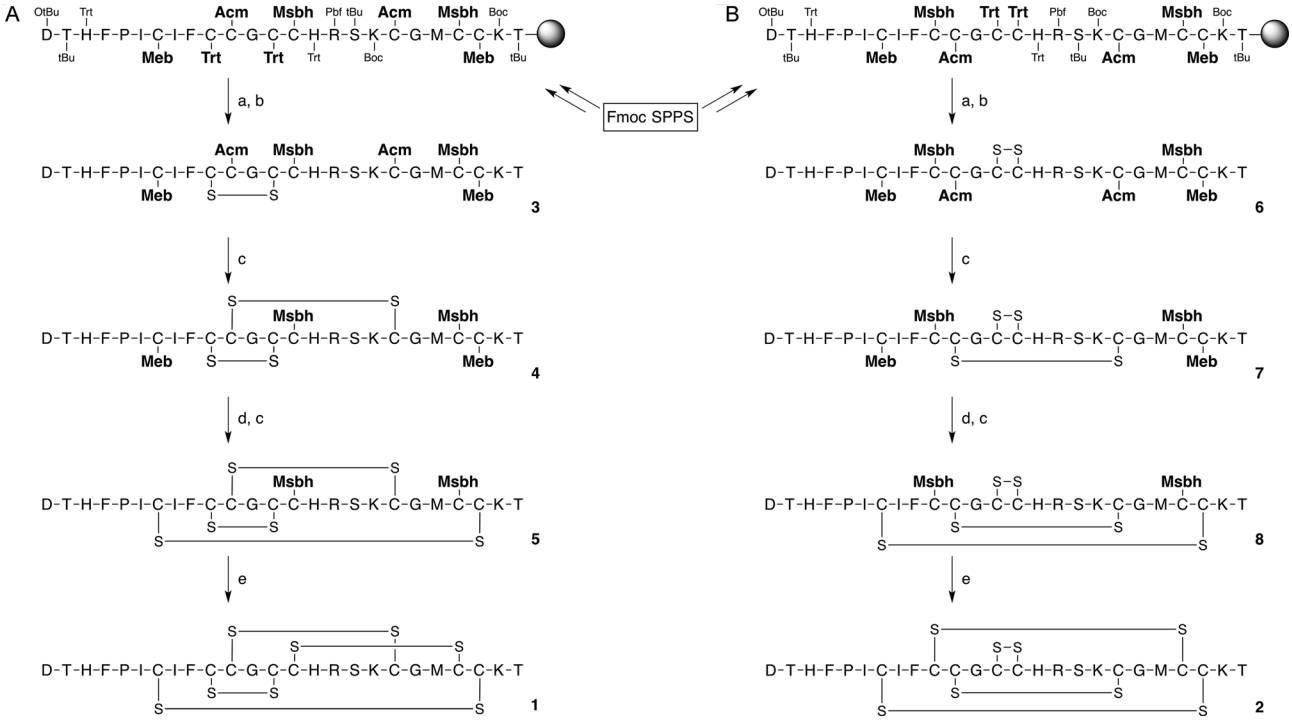
Initially, we prepared both N^{α}-Fmoc- and N^{α}-Boc-Cys-(Msbh)-OH (see the Supporting Information, p 5) and evaluated their broad suitability for peptide synthesis with both Boc- and Fmoc- chemistry using oxytocin (CYIQNCPLG; see the Supporting Information, p 12), which contains a single disulfide bond. For the reductive acidolytic cleavage of the Msbh group, we chose to employ the NH_4I/dimethylsulfide (DMS)/TFA system because its compatibility has been demonstrated with a range of cysteine-containing peptides.[12] Iodine or iodosulfonium ions produced during this reaction oxidize the liberated thiols to disulfides[12b] (see the Supporting Information, p 12), a convenient byproduct since our aim is the formation of disulfide bridges. While broadly applicable, a limitation of these deprotection conditions for use in tryptophan-containing peptides is discussed in the Supporting Information (p 34).

Next, Cys(Msbh) was applied with paired Cys(Trt), Cys-(Acm), and Cys(Meb) to the regioselective synthesis of the disulfide bonds of human hepcidin (**1**, Scheme 1 A). Chain assembly was performed on a Thr(*t*Bu)-Wang resin by using Fmoc chemistry with HBTU/DIEA activation for couplings (DIEA = diisopropylethylamine). Cleavage of the protected peptide from the resin with TFA/TIPS/H_2O yielded Cys 1,8 (Meb)/Cys 3,6(Acm)/Cys 5,7(Msbh) hepcidin with free thiols at Cys 2 and 4; these were subsequently oxidized with aqueous DMSO to give **3**. Subsequent oxidative deprotection of the Cys 3,6(Acm) pair was accomplished with I_2 in 0.1% TFA/MeCN/H_2O to give **4**. The Meb groups of Cys 1 and 8 were removed with HF/*p*-cresol and the liberated thiols subsequently oxidized with I_2 in 0.1% TFA/MeCN/H_2O to give **5**. Alternatively, Cys(Meb) could be substituted with the more acid labile Cys(Mob) to circumvent the need for the HF cleavage step, in which case trifluoromethanesulfonic acid (TFMSA)/TFA/*p*-cresol was used to remove the Mob group (see the Supporting Information, p 19). The Msbh groups remained intact throughout the course of these treatments. The treatment of **5** with NH_4I/DMS/TFA resulted in complete removal of the Msbh groups of Cys 5 and 7 with simultaneous disulfide-bond formation to give **1** as the single major product without significant byproducts (Figure S2 in the Supporting Information).

A vicinal disulfide form of hepcidin (**2**) was then synthesized with the alternate connectivity of Cys 1–8, 2–7, 3–6, 4–5[2a] by using an analogous strategy as outlined in Scheme 1 B. Product **1** co-eluted by HPLC with a commercial sample of human hepcidin (PI 4392s), which has the native fold and was prepared by the reported procedure[13] (see the Supporting Information, p 22), whereas product **2** eluted 14 seconds earlier (Figure 1).

Structural analysis of **1** by NMR spectroscopy revealed that the cysteine residues in particular were substantially broadened. This is a common observation in disulfide-rich peptides because the disulfide bonds connect distal segments of the peptide chain, which often undergo uncorrelated motion.[14] This conformational plasticity around disulfide bonds exacerbates an already difficult problem and as anticipated, the disulfide-bond connectivity of **1** could not be assigned from the 2D NMR experiments alone. However, by utilizing the knowledge of the disulfide connectivity gained from the regioselective chemical synthesis, a high-resolution 3D structure of **1** could be determined by using the experimental NOE restraints and chemical-shift-based dihedral-angle restraints.[15] The structure was found to be in good agreement with the NMR and X-ray structures of native



Scheme 1. Regioselective synthesis of hepcidins **1**[2b] (A) and **2**[2a] (B) by using Trt, Acm, Meb, and Msbh protecting groups. a) 95% TFA/2.5% TIPS/2.5% H_2O, b) 30% DMSO/5% AcOH/H_2O, c) I_2, 0.1% TFA/50% MeCN/H_2O, d) HF/*p*-cresol (9:1), e) NH_4I (40 equiv), 1% DMS/TFA. TIPS = triisopropylsilane, DMSO = dimethyl sulfoxide.

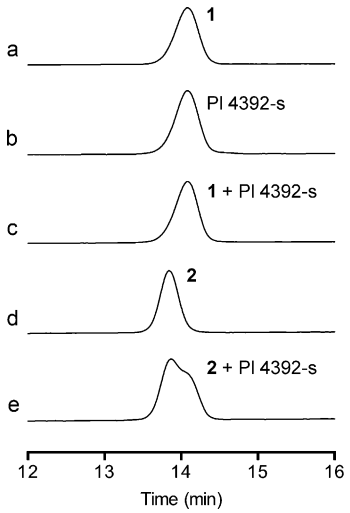


Figure 1. Analytical HPLC traces of **1** (a), commercial human hepcidin sample (Peptides International, PI 4392s; b), co-injection of **1** and PI 4392s (c), **2** (d), and co-injection of **2** and PI 4392s (e).

human hepcidin obtained by Jordan et al.;[2b] the core regions (residues 7–23) overlaid with rmsd values of 0.90 Å and 0.74 Å, respectively (Figure 2). Previous NMR-based structural studies have been forced to extreme temperatures (263 K or 325 K) to alleviate the difficulties associated with the exchange processes proximal to the disulfide bonds; however, we demonstrate herein that if the connectivity is known a priori, a high-resolution structure can be obtained even at ambient temperature (298 K). Indeed this structure is in better agreement with the crystal structure than the previous NMR structure at 325 K.[2b] Interestingly, the NMR spectra of **2** indicates the presence of multiple conformations. This non-native fold appears to be highly unstable and even at 323 K, the conformational exchange could not be averaged on the NMR time scale, thus making structure calculation unfeasible. Comparison of the ^{1}H–^{15}N and ^{1}H–^{13}C HSQC spectra of the two compounds revealed little overlap, thus indicating that none of the conformations of **2** are consistent with the fold of **1** (Figure S4). Since a single major product was obtained after each disulfide-forming step and **2** appears as a single symmetrical peak by analytical HPLC, it is unlikely that the multiple conformations seen by NMR are due to the presence of undesired disulfide-bond isomers. Nevertheless, to discount this possibility, a tryptic digest was performed. Each of the products appeared as a single peak of correct mass by analytical HPLC (see the Supporting Information, p 27). Additionally, fractionation of **2** gave peaks of correct mass that co-eluted on re-injection (see the Supporting Information, p 27).

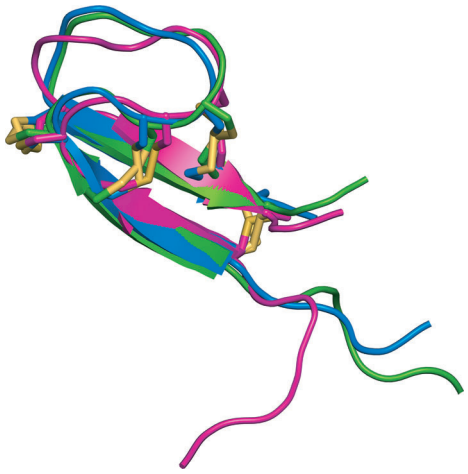


Figure 2. Superposition of the NMR structure of **1** obtained at 298 K (green) with that of native hepcidin at 325 K (pink, PDB ID 2KEF) and the X-ray structure of native hepcidin (blue, PDB ID 3H0T). Disulfide bonds are shown in yellow.

The hepcidin–ferroportin interaction is thought to involve a thiol–disulfide interchange between ferroportin thiol C326 and the hepcidin disulfide framework.[16] We assessed the bioactivity of the two regioselectively synthesized forms of human hepcidin through the quantitation of ferroportin–GFP degradation.[17] Forms **1** and **2**, as well as native human hepcidin (PI 4392s), stimulated the internalization and degradation of ferroportin–GFP with comparable EC_{50} values of 2.0, 4.4, and 3.9 nM respectively (see the Supporting Information, p 30). It was previously demonstrated that the nine-residue N-terminal fragment of hepcidin retains some bioactivity provided that there is at least one cysteine residue present.[16] This fact, taken together with the equipotency of **1** and **2**, may suggest that the native disulfide framework of hepcidin is not essential for its bioactivity. Alternatively, it is conceivable that the C326 thiol of ferroportin or other cell surface thiols induce disulfide-bond rearrangement of **2** to the native isomer **1**. Indeed we found that catalytic amounts of reduced glutathione are sufficient to effect the conversion of **2** into **1** (see the Supporting Information, p 31), thus suggesting that the fold of **1** is thermodynamically favored over that of **2**, something that may contribute to the conformational instability of **2** observed by NMR. To investigate the possibility of **2** rearranging into **1** under the conditions of the biological assay, **2** was incubated in the presence of cells expressing a non-internalizing ferroportin mutant[18] and the supernatant was analyzed by HPLC. Form **2** again rearranged into **1** (see the Supporting Information, p 32), therefore it was not possible to conclude whether **2** is indeed equipotent with **1** or whether the activity observed is due to **1** being generated from **2**.

In conclusion, the complete regioselective synthesis of two disulfide-bond isomers of hepcidin was achieved by using the newly developed Msbh thiol protecting group. We were able to rule out **2** as a possible structure of native hepcidin and provide further support for **1** as the native molecule with the correct S–S connectivity. Moreover, Msbh thiol protection expands the general capacity for the regioselective synthesis of peptides with multiple disulfide bonds by employing either Fmoc or Boc chemistry and complements the existing set of sulfoxide-type safety-catch protecting groups for multidimensional protection in peptide synthesis.

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