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Hydrazine-Sensitive Thiol Protecting Group for Peptide and Protein Chemistry

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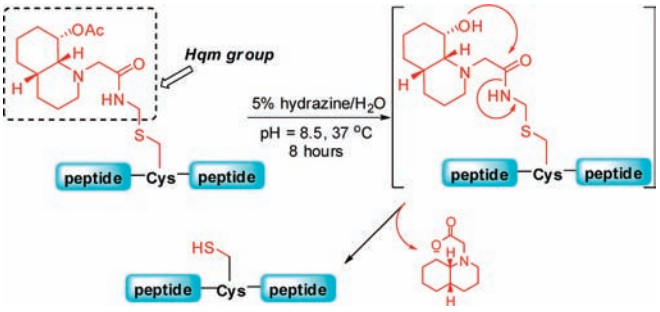
ABSTRACT



In the search for a new Cys side-chain protecting group that is compatible to the solid-phase peptide synthesis yet can be removed under mild conditions, the Hqm and Hgm groups that are readily deprotected by using aqueous hydrazine have been developed. The utility of these groups for peptide and protein chemistry is tested by the total synthesis of a peptide antibiotic trifolitoxin and the human neutrophil defensin hNP2.

Chemically synthesized peptides[1] and proteins[2] are important tools in chemical biology. The development of more efficient methods for the preparation of increasingly complex peptides and proteins still provides important challenges.[3] One such challenge is the synthesis of multiple Cys-containing peptides, where an often encountered difficulty is unambiguous formation of several disulfide bridges.[4] To solve this difficulty there is often a need to conduct orthogonal protection of Cys residues for the stepwise formation of disulfide bonds.[5] Second, for chemical synthesis of proteins without suitable Xaa-Cys ligation site(s), there is a need to use the recently developed "ligation–desulfurization" approach[6] in which the desulfurization step is performed in the presence of side chain protected Cys.[7] Finally, peptide–protein bioconjugation may also need selective protection and deprotection of active Cys side chains under mild conditions.[8]

In this context, it is important to stress that very few good-quality protecting groups are available for the Cys side chain.[9] The popular trityl (Trt) group[9] cannot survive the peptide cleavage step in SPPS (solid-phase peptide synthesis), whereas the disulfide protection (e.g., S^{t}Bu)[10] is not stable to the reductive conditions often used to handle

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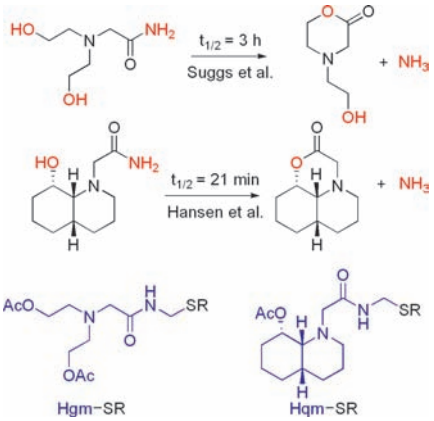


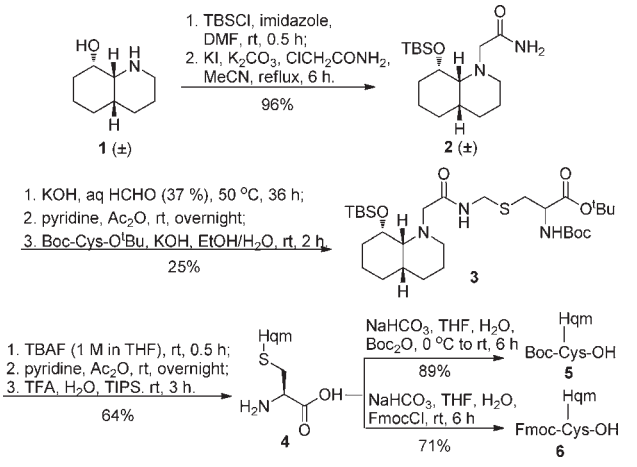
Figure 1. Design of new thiol protecting groups.

peptides. The ^{t}Bu and MeBzl (4-methylbenzyl) protecting groups have been shown to be useful in the stepwise formation of disulfide bridges,[5,11] but their deprotection requires harsh conditions. For practical purposes, the method of choice is the use of an acetamidomethyl (Acm) group[7,12] that is compatible with both *tert*-butoxycarbonyl (Boc) and 9-fluorenylmethyloxycarbonyl (Fmoc) SPPS. However, the deprotection of Acm either needs to use toxic heavy metals[7,12] or may cause side reactions (e.g., iodination at the Trp and Tyr residues).[13] Thus, new protection groups for the Cys side chain are still needed not only to overcome the problems associated with Acm but also to permit a more adaptable manipulation of Cys residues.

In our studies on protein chemical synthesis,[14] we question the possibility of developing a new Cys side chain protecting group that can be removed by aqueous hydrazine. Previous studies on protein chemistry have shown that the aqueous hydrazine condition provides a mild and operationally simple method for the removal of ester protection of alcohols.[15] The hydrazine method is also compatible with SPPS and ligation chemistry as demonstrated in the synthesis of full-length glycoproteins such as diptericin.[15] Thus, we envisage that a hydrazine-sensitive, yet stable-to-SPPS, thiol protecting group would be highly useful for peptide and protein chemistry. However, the previously developed thiol protecting groups[9] either cannot be removed by hydrazine or are unstable to SPPS.

To solve the above problems, we conjecture that the new protecting group can exploit the Acm strategy by using an acylamidomethyl group that is stable to SPPS. As a consequence, the challenge becomes to design a hydrazine-based approach to rupture the amide bond in the acylamidomethyl moiety. Noting the difficulty of amide bond cleavage under mild conditions, we turn to the idea of using intramolecular acyl transfer to promote the deprotection reaction. Previous studies by the groups of Suggs[16] and Hansen[17] provide interesting examples for rapid cleavage of unactivated amide bonds at neutral pH (Figure 1). Accordingly, we designed Hgm and Hqm groups (nomenclature for Hgm and Hqm is explained in the Supporting Information) for the protection of a thiol (Figure 1), in which the OAc moiety is removed by aqueous hydrazine to trigger the deprotection event.

Scheme 1. Synthesis of Hqm-Protected Cys



The synthesis of Hqm-protected Cys is illustrated in Scheme 1. The previously made **1**[17,18] (as a racemic mixture) is converted to **2** through TBS protection and *N*-alkylation. Then a key step is the reaction of **2** with formaldehyde to form an *N*-(hydroxymethyl)acylamide intermediate, which without separation is converted to the corresponding acylamidomethyl acetate. Nucleophilic substitution of the OAc group by Boc-Cys-O^{t}Bu affords **3**, which is converted to **4** through standard protecting group transformations. Finally, **4** is converted to Boc-Cys(Hqm)-OH (**5**) and Fmoc-Cys(Hqm)-OH (**6**). Note that **3**−**6** are synthesized as inseparable diastereomeric mixtures. Below we will show that these diastereomeric mixtures do not interfere with their applications in peptide and protein chemistry. Besides,

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Table 1. Solid-Phase Peptide Synthesis Using Hgm- and Hqm-Protected Cys

target peptides	SPPS method	yield %[a]
HgmS Ala-Phe-Lys-Ala-Cys-Lys **9**	Boc	73
HgmS Ala-Phe-Lys-Ala-Cys-Lys **10**	Boc	68
HgmS Gly-Gln-Lys-Ala-Ser-Gly-Cys-Tyr-Glu-Phe-His-Gly **11**	Fmoc	56
HqmS Val-Asp-Ile-Gly-Gly-Ser-Arg-Gln-Gly-Cys-Val-Ala **12**	Fmoc	58

[a] Isolated yields.

chiral resolution of **1** can be considered if enantiomerically pure **5** or **6** is needed. By using a similar synthetic route we have also prepared Boc-Cys(Hgm)-OH (**7**) and Fmoc-Cys(Hgm)-OH (**8**) (Supporting Information).

With the protected Cys **5**−**8** in hand, we next examined their compatibility with SPPS by synthesizing model peptides **9**−**12**. Both the Boc and Fmoc methods are tested (Table 1), and the results indicate that the Hgm and Hqm groups can well tolerate the standard SPPS conditions. Subsequently, we examined the deprotection of the Hqm group in model peptide **12**. At pH 8.5 and 37 °C, addition of 5% (v/v) hydrazine to the solution of **12** in Gn·HCl (guanidine hydrochloride) causes the removal of the Hqm group (Figure 2). The deprotection is complete in approximately 8 h with a HPLC yield of 93%. Note that **12** is a diastereomeric mixture due to the use of racemic mixture of **1** in the beginning of synthesis. Nonetheless, both the diastereomers transform to **13** with a similar speed.

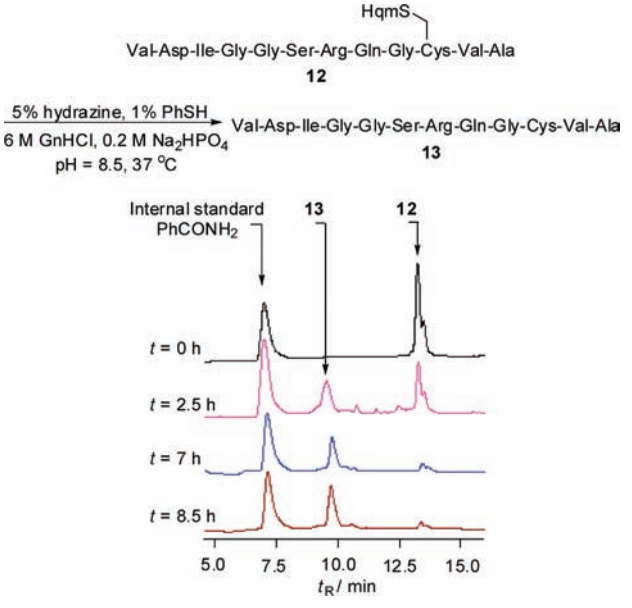

Figure 2. Deprotection of Hqm.

Compared to Hqm, the deprotection of Hgm is much slower. For model peptide **11**, use of the above conditions to remove the Hgm group requires three days to complete the reaction to give the target peptide in 91% yield. This observation is consistent with the previous findings by Suggs et al.[16] and Hansen et al.[17] shown in Figure 1. Thus, for practical purposes, the Hqm group is more useful. Furthermore, we have also tested the deprotection of Hqm in peptide **12** by using the traditional I_2 and AgOAc methods

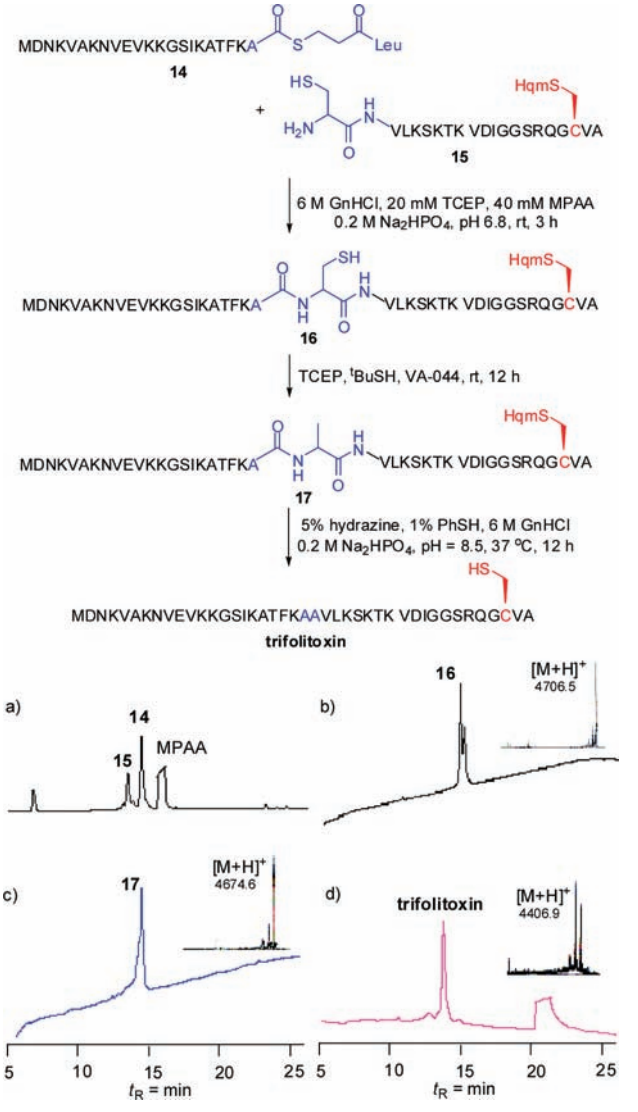

Figure 3. Synthesis of trifolitoxin and the corresponding analytic HPLC data. Observed and calculated masses for each peptide are as follows: **16** obsd = 4706.5 Da, calcd = 4704.6 Da; **17** obsd = 4674.6 Da, calcd = 4672.5 Da; trifolitoxin obsd = 4406.9 Da, calcd = 4406.2 Da. More details can be found in the Supporting Information.

developed for Acm.[12,13] Both these methods can deprotect Hqm to give **13** in over 90% yields in about 30 min. On the contrary, our test shows that the Acm group in Boc-Cys(Acm)-−OH is completely inert to the hydrazine conditions. Thus, Hqm is partially orthogonal to Acm, but Hqm is more flexible than Acm in the deprotection.

To test the application of the Hqm group in protein ligation chemistry, we chose trifolitoxin as the synthetic target which is a 42-mer peptide antibiotic.[19] Trifolitoxin has a single Cys40 residue very close to C-terminal so that this Cys is not suitable for the ligation. Therefore, we have to use the "ligation−desulfurization" approach[6] by changing Ala23 to Cys23 first. After the ligation is accomplished, Cys23 must be converted back to Ala23 and during this process Cys40 should remain intact. In our synthesis, Cys40 is protected by the Hqm group (Figure 3) in the peptide fragment **15** that is prepared through Fmoc SPPS. Native chemical ligation of **15** with trifolitoxin[1−22]-$^{\alpha}$thioester **14** generates a 42-mer peptide **16** in 90% yield. Desulfurization is then conducted to convert Cys23 back to Ala23 in 85% yield by using the metal-free reduction protocol developed by Danishefsky et al.[7] Finally, the desulfurized product **17** is treated with aqueous hydrazine at pH 8.5 for 12 h to produce the designed full-length trifolitoxin in 88% yield. Thus, the Hqm group provides another useful protecting group to be used in the "ligation−desulfurization" approach for protein chemical synthesis.[6]

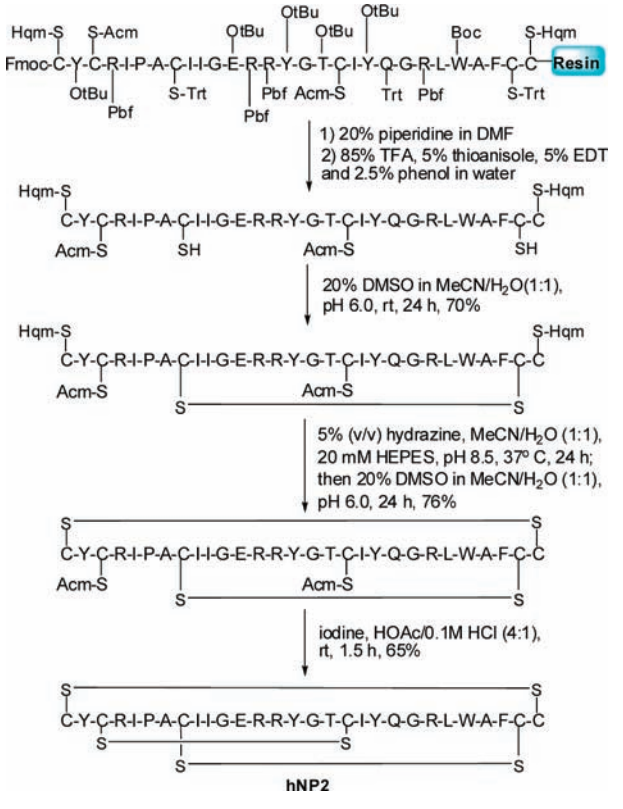

Figure 4. Use of Hqm in the synthesis of hNP2.

Having shown that Hqm can replace Acm in protein ligation chemistry, we also want to emphasize that Hqm can provide complementary utility that is not achievable with Acm alone. This feature can be demonstrated by the synthesis of human neutrophil defensin hNP2, which is a 29-mer peptide with three disulfide bridges.[20] A previous chemical synthesis of hNP2 by Raj et al.[21] used three orthogonal thiol protecting groups, namely, Trt, S^{t}Bu, and Acm. In our new synthesis, we use Trt, Hqm, and Acm as the thiol protecting groups (Figure 4). The Trt groups on Cys8 and Cys28 are removed when the peptide is cleaved from the resin. The Cys8−Cys28 disulfide bridge is then formed by using the DMSO oxidation in 70% isolated yield determined by weight of the peptide. Subsequently, the Hqm groups on Cys1 and Cys29 are removed by using aqueous hydrazine. This step is followed by DMSO oxidation to generate the Cys1−Cys29 disulfide bridge in 76% isolated yield. Finally, the Acm groups on Cys3 and Cys18 are removed by using iodine and during this process the Cys3−Cys18 disulfide bridge is generated in 65% isolated yield. The final product compares identical with the commercial hNP2 in both HPLC and MS analysis.[22] The overall yield of the three steps for disulfide formation is 34%. By comparison, in Raj's synthesis[21] the three disulfide bonds were formed in 68%, 58%, and 48% yields with an overall yield of 19%.

In summary, in the search for a new Cys side chain protecting group that is compatible to the solid-phase peptide synthesis yet can be removed under mild conditions, we developed the Hqm and Hgm groups that are readily deprotected by using aqueous hydrazine at pH 8.5. These groups provide more options for the manipulation of Cys residues in complex peptides and proteins, as demonstrated by the synthesis of a 42-mer peptide antibiotic trifolitoxin through the "ligation−desulfurization" approach. We also showed a new and high-yielding synthesis of human neutrophil defensin hNP2 using Hqm through stepwise formation of three disulfide bridges. These results show the value and potential of developing a hydrazine-sensitive yet stable-to-SPPS thiol protecting group. Further optimization and applications of the hydrazine-sensitive thiol protecting groups are being explored in our laboratory to improve the technology for complex peptide and protein synthesis.

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Supporting Information Available. Experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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(22) Note that the deprotection of Hqm at pH 8.5 may cause disulfide-bond isomerization. Our HPLC analysis indicated that this did not occur in the synthesis of hNP2. Detailed enzymatic degradation analysis on this problem will be reported in our ensuing studies.