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Chemoselective Ligation of Peptide Phenyl Esters with N-Terminal Cysteines

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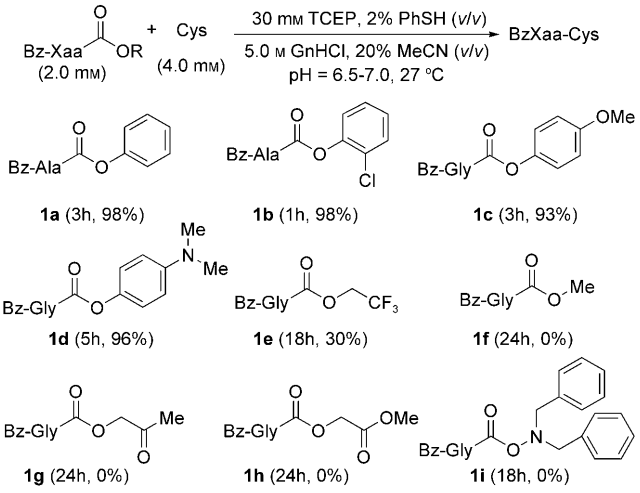
Protein chemical synthesis enables a level of control on protein composition beyond what is attainable with protein expression. It can provide otherwise inaccessible insights into protein's structure and function.[1] The development of increasingly efficient and general methods for the ligation of complex peptide fragments remains a central objective. The method of choice is a Cys-based native chemical ligation (NCL) developed by Kent et al.[2] NCL involves a chemoselective ligation reaction between a C-terminal thioester and an N-terminal Cys to yield a native peptide bond. Although being an extremely powerful method, the applicability of NCL might sometimes be limited in two respects: 1) The ligation reaction is restricted to the Cys residue. Thus, in some cases special techniques must be implemented by using a removable thiol-containing auxiliary, for example.[3] 2) The rate of NCL is strongly dependent on the C-terminal amino acid. Ligations at sterically hindered C-terminal sites, such as Val, Ile, and Pro, are very slow and the corresponding yields are often low.[4]

To help solve the second problem, Danishefsky and co-workers recently developed a highly interesting oxo-ester peptide ligation method.[5] Through the use of a fairly activated C-terminal *para*-nitrophenyl ester, it is possible to achieve direct Cys ligations. Importantly, peptide substrates incorporating bulky C-terminal amino acids can be accommodated with high reaction efficiency. The success of this novel concept of peptide ligation opens new opportunities for studies on protein chemical synthesis. Nonetheless, Danishefsky's pioneering method has two drawbacks that require further improvement: 1) undesired hydrolysis of the activated peptide *para*-nitrophenyl ester is sometimes a major competing reaction; 2) a direct solid-phase synthesis of peptide *para*-nitrophenyl ester remains very difficult.

Herein we report that by using simple and less-activated phenyl esters of peptides, chemoselective ligation between two unprotected peptide fragments can also proceed directly and smoothly under the promotion of imidazole.[6] Significantly, it is found that ligations at sterically hindered C-terminal sites (e.g., Val, Ile, Pro) are fairly efficient under the new conditions. Moreover, the previous problems associated with the *para*-nitrophenyl esters (that is, fast hydrolysis and difficult solid-phase synthesis) are successfully overcome by the new approach. Note that in earlier studies Kemp et al. and some other groups showed the use of phenyl ester derivatives in peptide couplings.[7,4c] Such couplings are different from the ligation described in this study because the present reaction requires the assistance of thiol capture and imidazole promotion, whereas the previous couplings were direct aminolysis reactions.

Our study began with the examination of a number of peptide *oxo*-esters for the Cys ligation in model systems (Scheme 1). It was found that most *oxo*-esters could not provide any ligation product. Surprisingly, when the phenyl ester (**1a**) was tested, we obtained a very high ligation yield (98%) in only 3 h. A more electron deficient phenyl ester (**1b**) reacted faster, whereas electron rich phenyl esters (**1c**, **d**) reacted slightly more slowly. Thiophenol was used as a promoter in the above ligations, but the ligation did not proceed well with the phenyl esters of more sterically hindered amino acids (e.g., Ile or Val).



Scheme 1. Ligation efficiency (HPLC yields) of different *oxo*-esters. Bz = benzoyl; TCEP = tris(2-carboxyethyl)phosphine; GnHCl = guanidine hydrochloride; MeCN = acetonitrile.

To search for possible improvements, imidazole was added to the ligation media.[8] To our surprise, we found that when the imidazole concentration was 2.5 M, the thiophenol promoter was no longer needed (Table 1). More interestingly, both sterically unhindered and hindered amino acids could be successfully ligated with Cys under the new conditions. For sterically unhindered amino acids the ligation was very fast (1–2 h) and the yields were very high (> 96%; entries 1–4). For the sterically demanding amino acids (Val, Ile, Pro), the ligation was slower (7–10 h) but the yields remained good (70–94%). In

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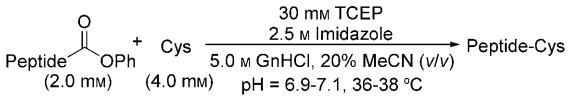
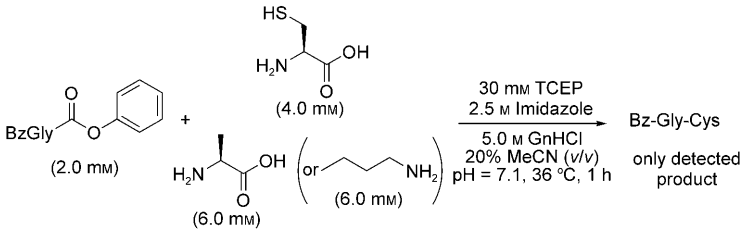
Table 1. Ligation (HPLC) yields of different amino acid esters.



	Peptide-	*t* [h]	Yield [%]
1	Bz-Gly- (**2a**)	1	99
2	Bz-Ala- (**2b**)	1	97
3	Bz-Met- (**2c**)	1	96
4	Bz-Phe- (**2d**)	2	96
5	Bz-Val- (**2e**)	7	94
6	Bz-Gly-Ile- (**2f**)	10	89
7	Bz-Gly-Pro- (**2g**)	10	70

comparison with these data, the ligation of peptide *para*-nitrophenyl esters generally took 2–15 h with a yield of 50–80%,[5] whereas the ligation between the thioester of Ile and Cys was reported to take 72 h to generate the product in 58% yield.[5]

Interestingly, it was found that the traditional thioester-mediated NCL could not be accelerated under the same imidazole conditions. To evaluate the selectivity of the phenyl ester ligation for the Cys functionality, a competition experiment was conducted in which Bz-Gly-OPh was added to a solution containing both Cys and Ala (Scheme 2). Dipeptide Bz-Gly-Cys



Scheme 2. Reaction scheme of a competition experiment in which Bz-Gly-OPh was added to a solution containing both Cys and Ala. Dipeptide Bz-Gly-Cys was the only observed product.

was the only observed product (Supporting Information); this strongly suggested that prior thiol capture of Cys predominates in the ligation. Furthermore, a competition experiment was also carried out in which Bz-Gly-OPh was added to a solution containing both Cys and nBuNH$_2$ [Eq. 1]. Again Bz-Gly-Cys was the only observed product; this indicates that the side chain of Lys does not interfere the phenyl ester ligation. Finally, the potential for racemization of the C-terminal amino acid in the ligation was examined by analyzing the reaction between Bz-Gly-Ala-OPh and Cys. The HPLC data (Supporting Information) showed that less than 1% was racemized.

Kinetic measurements showed that in cases in which the concentration of BzGly-OPh was 2 mM, the ligation rate with 4 mM Cys was similar to that with 16 mM Cys (Figure 1A). However, when the imidazole concentration increased from 0.5 to 2.5 M (in the presence of 2 mM BzGly-OPh and 4 mM Cys), the ligation rate increased by about 3.4 times (Figure 1B). Moreover, we found that the ligation rate in the presence of 4 mM Cys was almost identical to the rate of imidazole-promoted hydrolysis of the phenyl ester in the absence of Cys (Figure 1C). These data indicated that the phenyl ester reacts first (in a rate-limiting step, see Scheme 3) with imidazole to form a highly reactive intermediate (presumably acyl imidazole). Then the intermediate reacts rapidly with Cys through thiol capture[9] or it is destroyed by H_2O when Cys is absent.

To apply the phenyl ester ligation to protein chemical synthesis, we developed a solid-phase method to prepare peptide phenyl esters (Scheme 4).[10] The 4-methylbenzhydrylamine (MBHA) resin was used to attach 4-hydroxyphenylacetic acid. Subsequently the peptide phenyl ester could be readily synthesized through standard Boc chemistry. Thus, compared to peptide *para*-nitrophenyl esters, an important advantage of peptide phenyl ester is that it can be prepared through solid-phase synthesis. Note that we have tried to use Fmoc chemistry to prepare peptide phenyl esters, but failed.

To test whether the new ligation reaction might constitute a practical way to chemoselectively condense unprotected peptide segments, model protein synthesis was conducted for hadrurin,[11] a basic antimicrobial peptide. Native hadrurin does not contain any Cys (Scheme 5), but to facilitate the synthesis Ala10 was mutated to Cys (which can be readily converted back to Ala after the ligation[3h, 12]). This created a ligation site of Ile-Cys. As shown in Figure 2, the peptide phenyl ester (**3**) was ligated

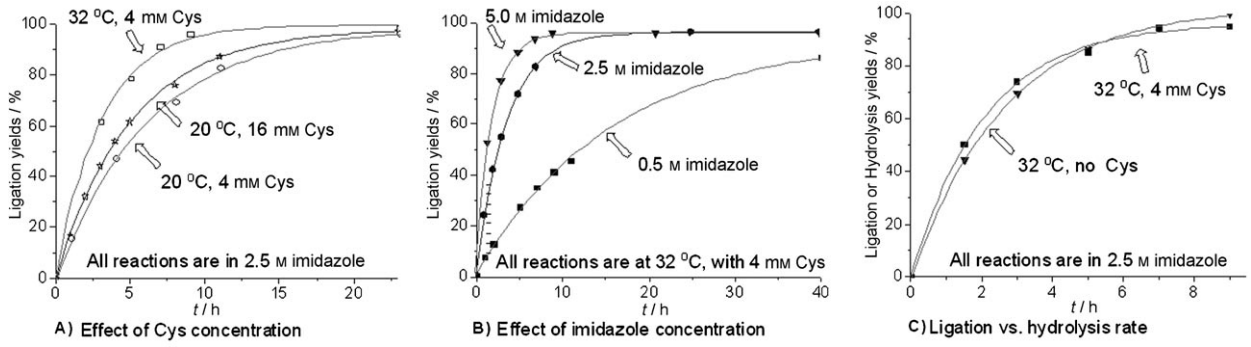
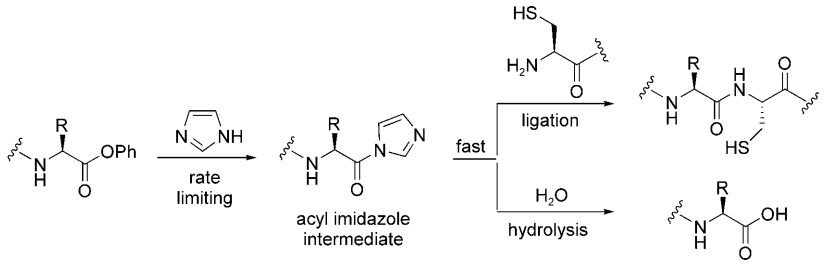
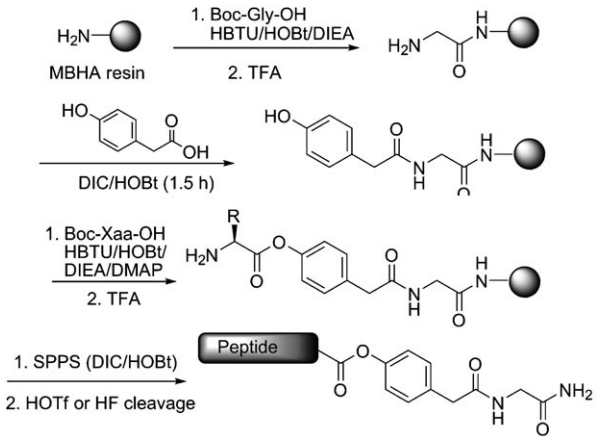


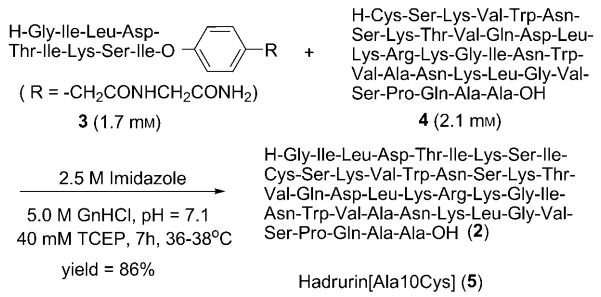
Figure 1. Kinetic data of phenyl ester ligation.



Scheme 3. Proposed mechanism of the phenyl ester ligation.



Scheme 4. Synthesis of peptide phenyl ester on the solid phase.



Scheme 5. Model synthesis of hadrurin.

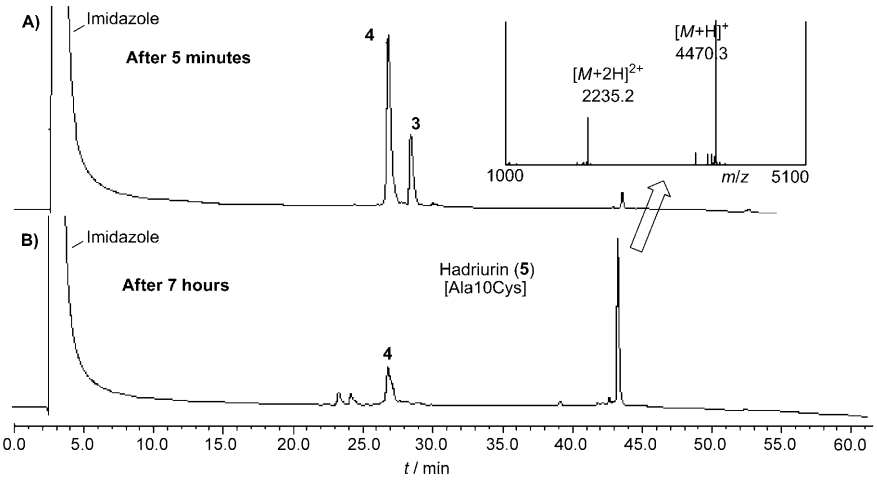
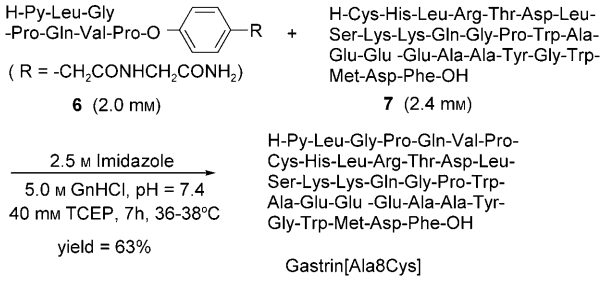


Figure 2. Analytic HPLC data for the ligation between **4** and **3**.

smoothly with peptide **4** to produce **5** (which was stable to NaOH treatment) in 7 h with a HPLC yield of 86%.

In the second example gastrin[Ala8Cys] was synthesized. Gastrin is a peptide hormone functioning as a physiological regulator of gastric acid secretion.[13] The ligation site was chosen to be Pro-Cys, an even more challenging position that has hardly ever been used in the traditional NCL. As shown in Scheme 6 and Figure 3, in cases in which the phenyl ester **6** was used, the Pro-Cys ligation was accomplished in 7 h with a HPLC yield of 63%. Both of the examples showed that the phenyl ester ligation is especially useful for the ligations at difficult sites.



Scheme 6. Model synthesis of gastrin.

To examine whether an internal Cys residue might interfere with the ligation,[14] we studied the reaction between Bz-Phe-OPh and a model peptide Cys-Gly-His-Cys-Tyr-Gly-Ala (Scheme 7). Only one product (which was stable to NaOH treatment) was obtained with a high yield (90%) and an expected mass (Supporting Information). Furthermore, we synthesized a peptide phenyl ester that contained an internal unprotected Cys (Scheme 8). The ligation of this peptide phenyl ester with Cys also provided one major product (which was stable to NaOH treatment) with a high yield (89%) and an expected mass (Supporting Information). Therefore, an internal unprotected Cys can be well tolerated in the ligation of peptide phenyl esters.

In summary, it was found that simple phenyl esters of peptides could undergo native chemical ligation smoothly under the promotion of imidazole. This new ligation proceeded rapidly at both sterically unhindered and hindered C-terminal sites (e.g., Val, Ile, Pro) to form the desired

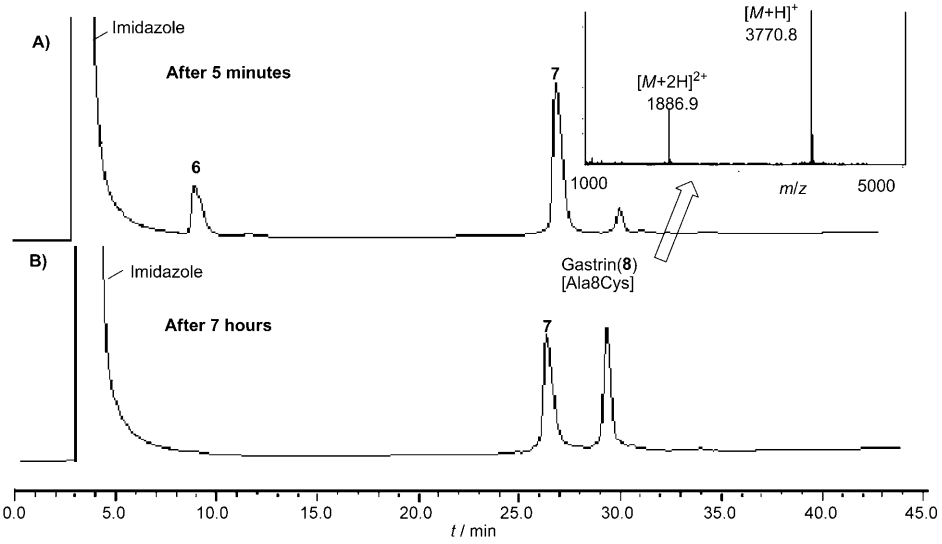
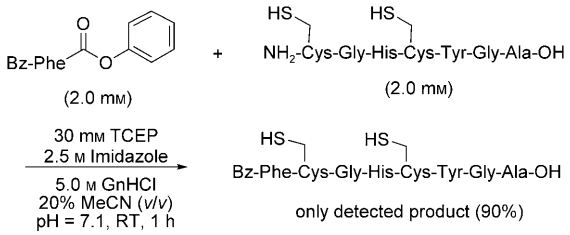
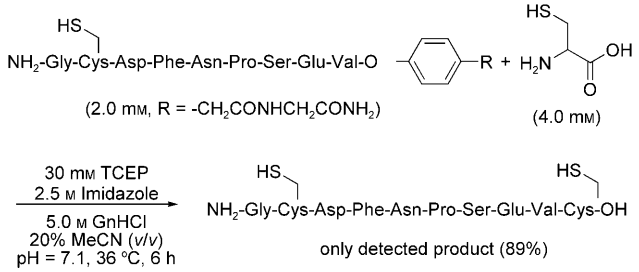


Figure 3. Analytic HPLC data for the ligation between **6** and **7**.



Scheme 7. Reaction between Bz-Phe-OPh and model peptide Cys-Gly-His-Gys-Tyr-Gly-Ala.



Scheme 8. Synthesis of a peptide phenyl ester containing an internal unprotected Cys. The ligation of this peptide phenyl ester with Cys provided one major product.

product in good to excellent yields. Peptide phenyl esters could be readily prepared through solid-phase synthesis as shown in the model protein synthesis of hadrurin[Ala10Cys] and gastrin[Ala8Cys]. The new method might constitute a useful method complementary to traditional native chemical ligation, especially for ligation at sterically demanding sites.

Experimental Section

Chemoselective ligation for the synthesis of hadrurin[Ala10Cys] [Eq. (2)]: Peptide phenyl ester **3** (0.6 mg, 0.52 μmol) was dissolved in an aqueous solution of Gn·HCl (6 M, 0.15 mL, containing 5 μL TFA). N-terminal Cys peptide **4** (1.7 mg, 0.482 μmol) was dissolved in the ligation buffer (0.113 mL, 5.0 M imidazole, 4.0 M Gn·HCl, 80 mM TCEP, pH 7.14, pH was adjusted by using neat TFA). The reaction mixture containing both the solutions of **3** and **4** (0.1 mL each, pH 7.10) was stirred at 36–38 °C under argon. The reaction was monitored by HPLC. The ligation product (**5**) was confirmed by MALDI-TOF with a HPLC yield of 86%.

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Keywords: native chemical ligation · peptide phenyl ester · peptides · solid-phase synthesis

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