Glycopeptides

DOI: 10.1002/anie.200702865

# Development of Efficient Methods for Accomplishing Cysteine-Free Peptide and Glycopeptide Coupling**

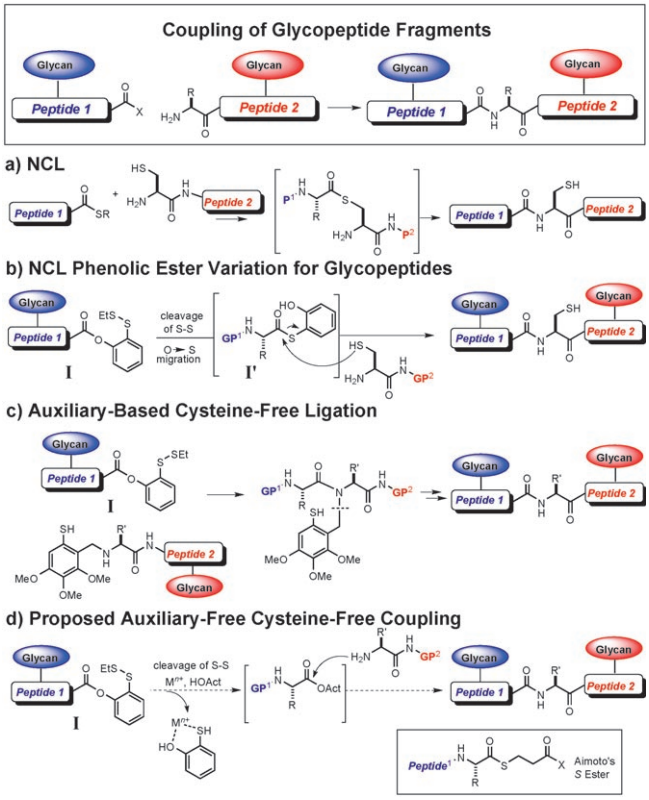
*Gong Chen, Qian Wan, Zhongping Tan, Cindy Kan, Zihao Hua, Krishnakumar Ranganathan, and Samuel J. Danishefsky**

We are currently pursuing the total synthesis of complex biologically relevant glycoproteins, bearing multiple oligosaccharide domains.[1] The scope and complexity of the problem prompts new thinking and new experimental designs. In addition to the learning opportunities which motivate the venture, there are specific glycoproteins of extraordinary biological activity and potency, which serve as targets for these investigations.[2] More broadly, potential therapeutic agents are viewed in pharma perspectives as either "small molecules" or "biologicals." The former are derived from chemistry while the latter are seen to emanate exclusively from biological means. Thus, our goal, as seen from a longer-range perspective, is that of reaching important "biologic"-level agents by chemical synthesis. If realizable, such a capability would bring at least some "biologics" under the purview of medicinal chemistry type optimization platforms.

A convergent synthetic strategy would involve the iterative coupling of individual glycopeptide fragments, each bearing one or more carbohydrate units.[3] The brilliant advance by Kent and co-workers, in using N-terminal cysteine residues as acyl acceptors in polypeptide–polypeptide ligations, served as the starting point for our studies (Scheme 1a).[4] Although the native chemical ligation (NCL) used by Kent and co-workers is now routinely applied in the synthesis of peptides and proteins, reliable methods for comparable ligations of glycopeptides bearing complex O-linked and N-linked glycan domains remained to be developed.[5]

In furtherance of our program, we recently devised and brought to fruition a new idea to accomplish the ligation of glycopeptide fragments. It is based on the use of a fairly stable phenolic ester bearing a protected *ortho*-thiol moiety (**I**) in place of the conventional thiol ester acyl donor in NCL (Scheme 1b).[6] The relatively inert nature of **I** provides an orthogonal possibility for ligations. The method works very nicely with N-terminal cysteine acyl acceptors. However, because of the paucity of cysteine residues in certain important natural protein sequences, methods to accomplish ligation to non-cysteine-based acyl acceptors are often required. [7,8] Toward that end, we also reported cysteine-free peptide-bond-forming protocols based on **I** in the broad context of our phenolic ester strategy (Scheme 1c).[9] However, while enabling the construction of homogeneous polypeptide oligosaccharides, the auxiliary-based approach may suffer from significant limitations in regard to required applications. First, the culminating S→N acyl transfer can be problematic when applied to the joining of relatively hindered aminoacyl moieties. Furthermore, detachment of the auxiliary with survival of vulnerable glycosidic bonds can be quite delicate.

To circumvent the issue of auxiliary removal, which is particularly worrisome in glycopolypeptide contexts




***Scheme 1.*** Coupling of glycopeptide fragments.

[*] Dr. G. Chen, Dr. Q. Wan, Dr. Z. Tan, Dr. C. Kan, Dr. Z. Hua, Dr. K. Ranganathan, Prof. S. J. Danishefsky
Laboratory of Bioorganic Chemistry
Memorial Sloan-Kettering Cancer Center
1275 York Avenue, Box 106, New York, NY 10021 (USA)
Fax: (+1) 212-772-8691
E-mail: danishes@mskcc.org
Prof. S. J. Danishefsky
Department of Chemistry
Columbia University
3000 Broadway, New York, NY 10027 (USA)

[**] This work was supported by the NIH (CA28824 to S.J.D) and the New York State Breast Cancer Research and Education Fund (C020919).

Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

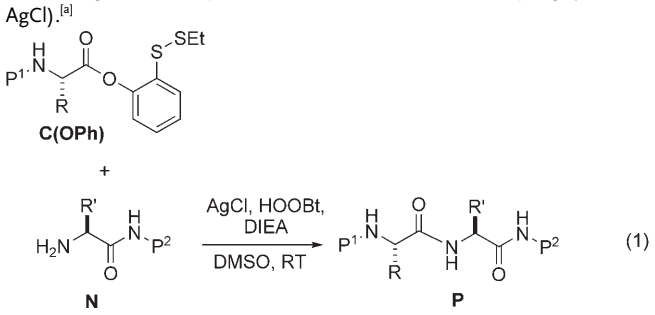
(Scheme 1c), we wondered about accomplishing a direct auxiliary-free coupling, wherein the N terminus of the general acyl acceptor fragment would be a free amine (not necessarily cysteine). Our in situ generated S-phenolic esters, derived from glycopolypeptide phenyl ester **I**, although susceptible to transthioesterification reactions[8] (see Scheme 1b,c), fails to couple with polypeptides with a non-cysteine terminal amine acyl acceptor. To solve this problem, we hoped to enhance the acyl donor capacity of **I** in the reaction.

The initiating factor for our study was the mechanistic logic of the known Blake–Aimoto silver ion mediated, fragment condensation reaction.[10] By analogy, we envisioned taking advantage of unique features of the juxtaposed *ortho*-SH phenol to facilitate the generation of a more-activated acyl donor. We explored the use of our type **I** esters in peptide–peptide fragment couplings.

Peptide substrates bearing C-terminal glycine or proline residues (which do not suffer from racemization problems) were used in these initial forays. Side chains were only protected at their Lys and Cys residues by ivDde (1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl) and Acm (acetamidomethyl), respectively;[11] all other functions remained free. DIEA/HOOBt (diisopropylethylamine/3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine) was used as the base/activator combination. DMSO was the preferred solvent, because of its anticipated strong solvating ability. A survey of different metal ions ($Cu^{+}$, $Cu^{2+}$, $Zn^{2+}$, $Ni^{2+}$, $Co^{2+}$, $Ag^{+}$) for the coupling of **1** and **2** (5 mM in DMSO) was conducted in the presence of 20 equivalents of DIEA, 25 equivalents of HOOBt, and 5 equivalents of tris(2-carboxyethyl)phosphine hydrochloride (TCEP·HCl) as a reducing agent for the disulfide.[12] Although metal ions which might have been expected to have exploitably high affinities for the *ortho*-SH phenol ensemble did not yield promising results, we were encouraged to observe (by LC-MS analysis) the formation of desired product **3** (ca. 30%) when the thiophilic $AgBF_4$ was used to mediate the reaction. We next evaluated the insoluble silver source AgCl, which had been used in the Aimoto system.[10] The coupling of **1** and **2** proceeded readily to give **3** in very good yields. Surprisingly, the reaction worked even more cleanly in the absence of the reducing agent TCEP·HCl [Eq. (1)]. Under this set of conditions, a variety of substrates were examined and excellent isolated yields were obtained in all cases (Table 1).

We conjecture, although without mechanistic specifics, that AgCl participates in the initial cleavage of the disulfide bond of the phenolic ester by somehow facilitating nucleophilic attack of HOOBt, initially present in excess.[13] The postulated priming of the disulfide bond of **1** by AgCl could well be the rate-determining step in the sequence, en route to the generation of the enhanced acyl donor (perhaps an HOOBt ester). In a valuable extension of this method, we have been able to prepare cyclic peptides in excellent yields without the need for dramatic substrate dilution (Table 1, entry 5).[14] The mechanism of the reductive phase of the apparent disproportionation, and a full account of the sulfur-containing products, remains to be accomplished.[15] Nonetheless, this novel route to synthetic cyclic and acyclic polypeptides is already a powerful new resource.

***Table 1:*** AgCl-assisted phenolic ester directed amide coupling (PEDAC AgCl).[a]




(1)

| Entry | Peptide substrates | *t* [h] | Yield [%] |
|---|---|---|---|
| 1 | **C**: FmocRNEDLS***G***OPh (**1**) | | |
| | **N**: $NH_2$LRK′DITC′G (**2**) | | |
| | **P**: FmocRNEDLS***GL***RK′DITCG (**3**) | 8 | 86 |
| 2 | **C**: FmocRNEDLS***G***OPh (**1**) | | |
| | **N**: $NH_2$***D***TK′VRFYAWK′RMEVG (**4**) | | |
| | **P**: FmocRNEDLS***GD***TK′VRFYAWK′R MEVG (**5**) | 12 | 82 |
| 3 | **C**: FmocRNEDLS***P***OPh (**6**) | | |
| | **N**: $NH_2$LRK′DITCG (**2**) | | |
| | **P**: FmocRNEDLS***PL***RK′DITCG (**7**) | 48 | 78 |
| 4 | **C**: FmocRNEDLS***P***OPh (**6**) | | |
| | **N**: $NH_2$***Q***RWIDTRG (**8**) | | |
| | **P**: FmocRNEDLS***PQ***RWIDTRG (**9**) | 48 | 75 |
| 5 | **N/C**: $NH_2$LRVIVADVFRKG***P***OPh (**10**) | | |
| | **P**: LRVIVADVFRK′G***P*** (**11**) | 48 | 81 |

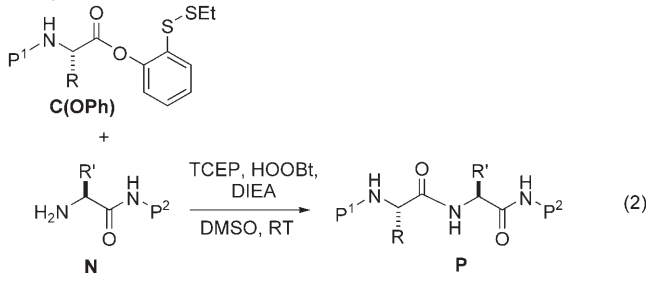
[a] General conditions: peptides 1–5 mM, AgCl (5 equiv), DIEA (20 equiv), HOOBt (25 equiv), DMSO, RT (K′: Lys(ivDde), C′: Cys(Acm)). Fmoc = 9-fluorenylmethoxycarbonyl. The coupled residues are in italics.

Subsequent control experiments revealed a novel and particularly valuable reaction pathway. Thus, the coupling of **1** and **4** in the presence of TCEP·HCl/HOOBt/DIEA proceeded unexpectedly well, even in the absence of the AgCl mediator [Eq. (2)]. With this finding, we have now discovered a powerful set of metal-free coupling conditions capable of transforming **1** to a high energy acyl donor form (possibly the HOOBt ester) that is capable of undergoing fragment–fragment coupling with a non-cysteine, free amine terminal acyl acceptor.

As shown below, under TCEP actuation, type **I** peptide esters bearing C-terminal Gly and Pro residues couple smoothly with peptidyl amines to afford adducts in excellent yields. Notably, the partially protected 64-mer **15** was obtained in 72% yield. Peptidyl esters bearing C-terminal Ala and Phe residues were also examined. A variety of conditions were screened in an effort to suppress racemization of the C-terminal residues. The sterically hindered base, 2,5-di-*tert*-butyl-*N*,*N*-dimethylaminopyridine (DBDMAP, $pK_a \approx 9$), provided the most promising results, although coupling rates and yields dropped slightly in comparison with DIEA (Table 2, entries 4 and 5).[16] Racemization of Ala (**16**) decreased from 12–15% to 7–9% and racemization of Phe (**18**) decreased from 40–45% to 20–25%. Thus, although some improvements with this method have been realized, complete suppression of the racemization at non-Gly or Pro sites remains an elusive goal.

***Table 2:*** TCEP-assisted phenolic ester directed amide coupling (PEDAC TCEP).[a]




(2)

| Entry | Peptide Substrates | *t* [h] | Yield [%] |
|---|---|---|---|
| 1 | **C**: FmocRNEDLS**G**OPh (**1**)<br>**N**: $NH_2$**D**TK′VRFYAWK′RMEVG (**4**)<br>**P**: FmocRNEDLSG***GD***TK′VRFYAW K′RMEVG (**5**) | 10 | 82 |
| 2 | **C**: FmocRNEDLS**P**OPh (**6**)<br>**N**: $NH_2$**D**TK′VRFYAWK′RMEVG (**4**)<br>**P**: FmocRNEDLS***PD***TK′VRFYAWK′R MEVG (**12**) | 24 | 78 |
| 3 | **C**: FmocLRSLTTLLRALGAQK′DAVSPP DAA**P**OPh (**13**)<br>**N**: $NH_2$**A**PLRTITADTFRK′LFRVYSNFLRGK′L K′LYTGEACRTGDR (**14**)<br>**P**: FmocLRSLTTLLRALGAQK′DAVSPPDAA ***PA***PLRTITADTFRK′LFRVYSNFLRGK′LK′ LYTGEACRTGDR (**15**) | 48 | 72 |
| 4 | **C**: FmocRNEDLSG**A**OPh (**16**)<br>**N**: $NH_2$**L**RK′DITC′G (**2**)<br>**P**: FmocRNEDLSG***AL***RK′DITCG (**17**) | 12<br>24 | 78<br>62[b] |
| 5 | **C**: FmocRNEDLSG**F**OPh (**18**)<br>**N**: $NH_2$**L**RK′DITC′)<br>**P**: FmocRNEDLSG***FL***RK′DITCG (**19**) | 12<br>24 | 76<br>61[b] |

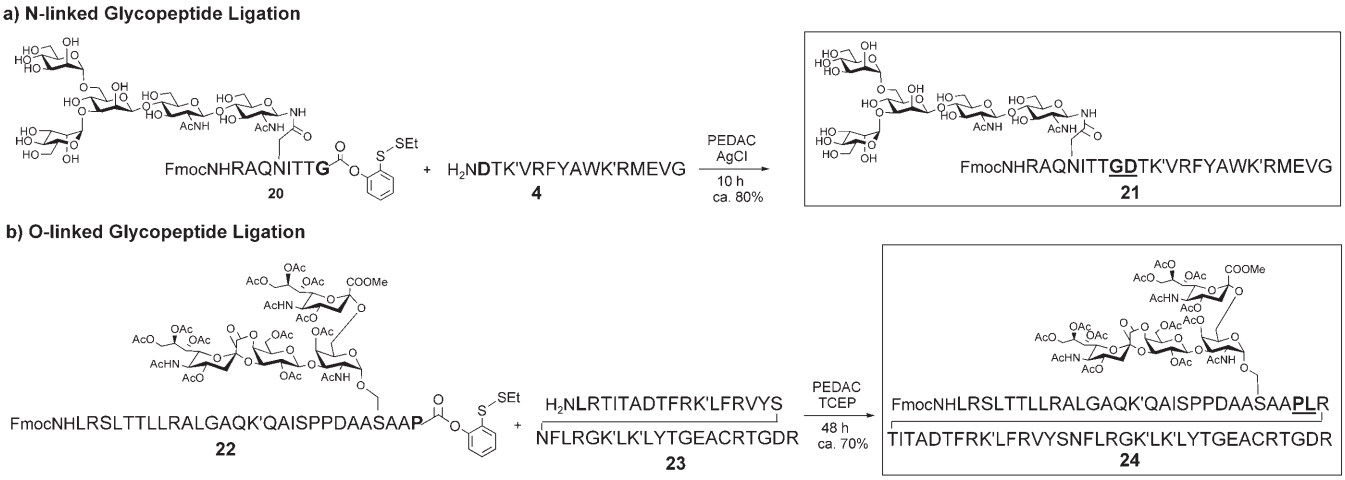
[a] General conditions: peptides 1–5 mM, TCEP·HCl (3 equiv), DIEA (25 equiv), HOOBt (25 equiv), DMSO, RT (K′: Lys(ivDde), C′: Cys(Acm)). [b] As above, except using DBDMAP as a base in place of DIEA.

Scheme 2 underscores that both the silver chloride and the TCEP-actuated fragment–fragment coupling reactions do indeed allow the synthesis of complex glycopeptides.[17] Thus, the 23-mer glycopeptide **21**, containing an N-linked pentameric glycan, as well as the 65-mer glycopeptide **24**, bearing a protected O-linked tetrasaccharide glycophorin moiety, were efficiently prepared using PEDAC methods, mediated by AgCl and TCEP·HCl, respectively (Scheme 2).

With both metal (AgCl) and metal-free (TCEP) actuation protocols now in place for the coupling of fragments of type **I** peptidyl and glycopeptidyl esters, we probed further to ascertain whether the two methods could be harmonized such as to allow for the process to be reiterated, including in two dimensions.[18] Indeed it proved possible to discover and exploit clear specificities in the maintenance and activatability of resident functional patterns. A particularly important finding was that TCEP activation of acyl donors is possible even in the presence of an alkyl thioester at the C terminus of the non-cysteine acyl acceptor. This is important since maintenance of a C-terminal alkyl thioester on the polypeptide (or glycopeptide) is clearly not feasible for the AgCl-mediated fragment coupling. The type **I** activatable phenyl ester donor functions well in the TCEP context even when carrying a substantial fully deprotected N-linked carbohydrate domain (Scheme 3a). With the TCEP-mediated coupling of **20** + **25** accomplished, we were well-positioned to use AgCl mediation to activate the alkyl thioester in product **26** for reiteration (in the C-terminal direction) by joining to an N-terminal acyl acceptor (see the formation of **27** in Scheme 3a). A powerful strategy for the synthesis of complex glycopeptide building blocks (for glycoprotein synthesis) from simple and more easily accessible precursors has now emerged.

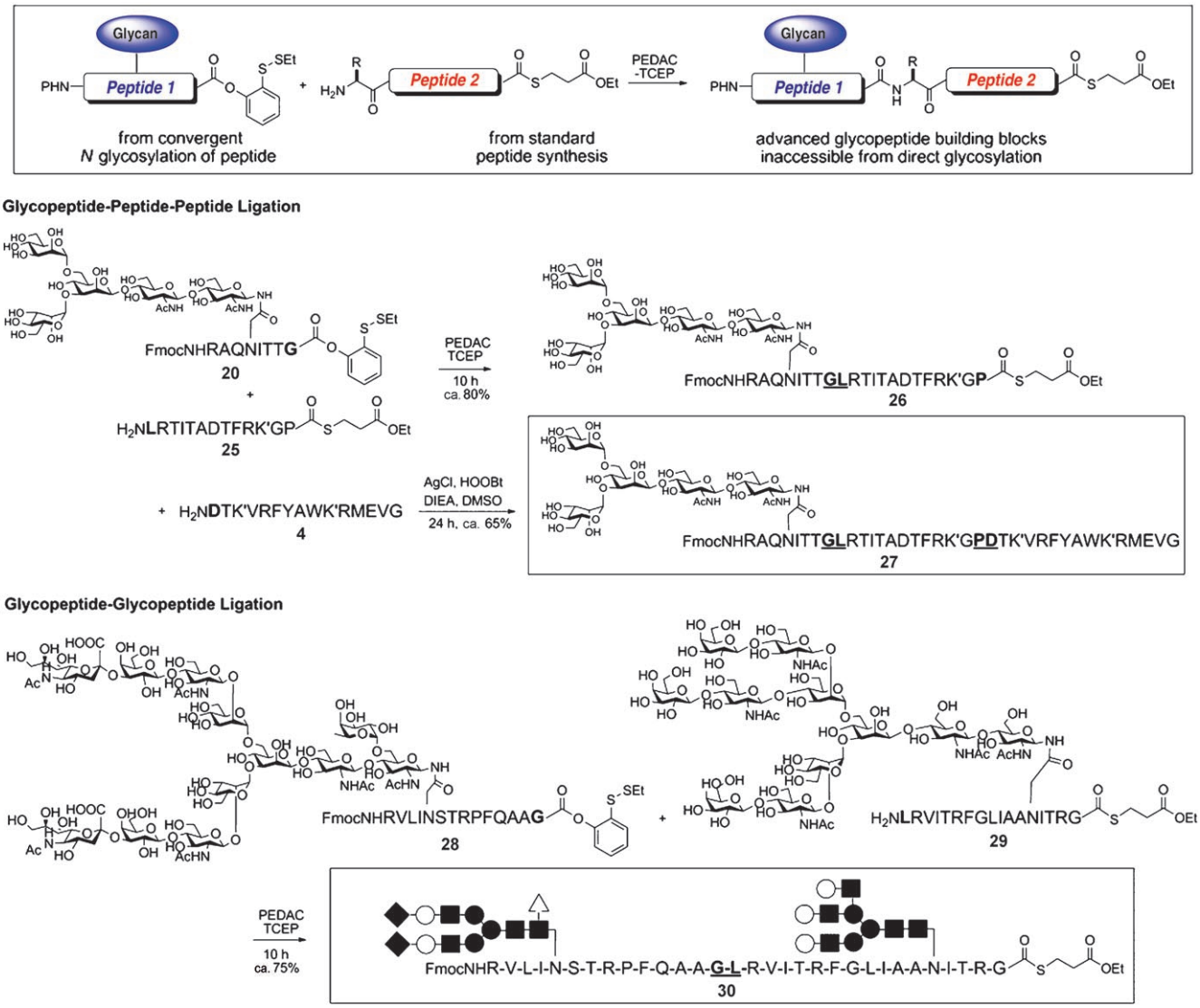
As a further demonstration of the power and reach of the methodology, a formidable bidomainal 31-mer glycopeptide containing one 12-mer and one 11-mer glycan was prepared in excellent yield (**30**, Scheme 3b). Conceivably, **30** could be further functionalized to generate even more complex targets.

In summary, given advances in the total synthesis of appropriately complex high-mannose entities required for N-linkage sites and given the cassette-based synthesis of the required O-linked glycophorin sectors which we developed some years ago, the oligosaccharides required for the




***Scheme 2.*** Glycopeptide synthesis.




***Scheme 3.*** Synthesis of highly complex glycopeptides.

program, while admittedly challenging, are accessible. The actual N-linkage step joining anomeric amine-based oligosaccharide acceptors to a differentiated aspartate residue in the initial peptidyl acyl donor, while still a work in progress from a process standpoint, is manageable. The ligation/fragment coupling possibilities, previously restricted to cysteine-based NCL and auxiliary-based, non-cysteine ligation, has now been expanded in important ways. In particular, the metal (AgCl) and metal-free (TCEP) mediated acyl donor enhancement of our recently developed type **I** C-terminal esters, provides a major expansion of options in pursuit of the total chemical synthesis of glycopolypeptide "biologics."[19]

Received: June 28, 2007
Published online: September 7, 2007

**Keywords:** coupling reactions · glycopeptides · glycoproteins · peptides · synthetic methods

[1] a) Z. Wang, X. Zhang, M. Visser, D. Live, A. Zatorski, U. Iserloh, K. O. Lloyd, S. J. Danishefsky, *Angew. Chem.* **2001**, *113*, 1778–1782; *Angew. Chem. Int. Ed.* **2001**, *40*, 1728–1732; b) J. S. Miller, V. Y. Dudkin, G. J. Lyon, T. W. Muir, S. J. Danishefsky, *Angew. Chem.* **2003**, *115*, 447–450; *Angew. Chem. Int. Ed.* **2003**, *42*, 431–434; c) B. Wu, Z. Tan, G. Chen, J. Chen, Z. Hua, Q. Wan, K. Ranganathan, S. J. Danishefsky, *Tetrahedron Lett.* **2006**, *47*, 8009–8011; d) J. Chen, G. Chen, B. Wu, Q. Wan, Z. Tan, Z. Hua, S. J. Danishefsky, *Tetrahedron Lett.* **2006**, *47*, 8013–8016.

[2] For example, a) erythropoietin: S. B. Krantz, *J. Am. Soc. Hemat.* **1991**, *77*, 419–434; b) follicle stimulating hormone (FSH): A. Ulloa-Aguirre, A. R. Midgley, Jr., I. Z. Beitins, V. Padmanabhan, *Endocr. Rev.* **1995**, *16*, 765–787.

[3] a) M. J. Grogan, M. R. Pratt, L. A. Marcaurelle, C. R. Bertozzi, *Annu. Rev. Biochem.* **2002**, *71*, 593–634; b) B. G. Davis, *Chem. Rev.* **2002**, *102*, 579–601; c) L. Liu, C. S. Bennett, C.-H. Wong, *Chem. Commun.* **2006**, 21–33.

[4] a) P. E. Dawson, T. W. Muir, I. Clark-Lewis, S. B. H. Kent, *Science* **1994**, *266*, 776–779; b) J. P. Tam, Y. A. Lu, C. F. Liu, J. Shao, *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 12485–12489;

c) R. M. Hofmann, T. W. Muir, *Curr. Opin. Biotechnol.* **2002**, 13, 297 – 303.

[5] a) D. Macmillan, C. R. Bertozzi, *Angew. Chem.* **2004**, *116*, 1379 – 1383; *Angew. Chem. Int. Ed.* **2004**, *43*, 1355 – 1359; b) C. P. R. Hackenberger, C. T. Friel, S. E. Radford, B. Imperiali, *J. Am. Chem. Soc.* **2005**, *127*, 12882 – 12889; c) A. Brik, Y. Y. Yang, S. Ficht, C.-H. Wong, *J. Am. Chem. Soc.* **2006**, *128*, 5626 – 5627.

[6] a) J. D. Warren, J. S. Miller, S. J. Keding, S. J. Danishefsky, *J. Am. Chem. Soc.* **2004**, *126*, 6576 – 6578; for a mechanistic study, see b) G. Chen, J. D. Warren, J. Chen, B. Wu, Q. Wan, S. J. Danishefsky, *J. Am. Chem. Soc.* **2006**, *128*, 7560 – 7562.

[7] a) J. Offer, P. E. Dawson, *Org. Lett.* **2000**, *2*, 23 – 26; b) D. W. Low, M. G. Hill, M. R. Carrasco, S. B. H. Kent, P. Botti, *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 6554 – 6559; c) J. Offer, C. N. C. Boddy, P. E. Dawson, *J. Am. Chem. Soc.* **2002**, *124*, 4642 – 4646.

[8] a) E. Saxon, J. I. Armstrong, C. R. Bertozzi, *Org. Lett.* **2000**, *2*, 2141 – 2143; b) M. B. Soellner, B. L. Nilsson, R. T. Raines, *J. Am. Chem. Soc.* **2006**, *128*, 8820 – 8828; c) J. W. Bode, R. M. Fox, K. D. Baucom, *Angew. Chem.* **2006**, *118*, 1270 – 1274; *Angew. Chem. Int. Ed.* **2006**, *45*, 1248 – 1252.

[9] B. Wu, J. Chen, J. D. Warren, G. Chen, Z. Hua, S. J. Danishefsky, *Angew. Chem.* **2006**, *118*, 4222 – 4231; *Angew. Chem. Int. Ed.* **2006**, *45*, 4116 – 4125.

[10] a) J. Blake, *Int. J. Pept. Protein Res.* **1981**, *17*, 273 – 274; b) S. Aimoto, N. Mizoguchi, H. Hojo, S. Yoshimura, *Bull. Chem. Soc. Jpn.* **1989**, *62*, 524 – 531; c) S. Aimoto, *Biopolymers* **1999**, *51*, 247 – 265.

[11] Protecting groups Acm for Cys and ivDde for Lys can survive the acidic deprotection of peptides and be easily removed by treatment with $Ag^{+}$, $Hg^{2+}$, or hydrazine under mild conditions.

[12] a) Y. A. Lu, J. P. Tam, *Org. Lett.* **2005**, *7*, 5003 – 5006; b) R. Ingenito, H. Wenschuh, *Org. Lett.* **2003**, *5*, 4587 – 4590.

[13] a) J. A. Pappas, *J. Am. Chem. Soc.* **1977**, *99*, 2926 – 2930; b) J. A. Pappas, *J. Am. Chem. Soc.* **1979**, *101*, 561 – 567.

[14] a) J. Chen, G. Chen, B. Wu, Q. Wan, Z. Tan, Z. Hua, S. J. Danishefsky, *Tetrahedron Lett.* **2006**, *47*, 1969 – 1972; b) L. Zhang, J. P. Tam, *J. Am. Chem. Soc.* **1997**, *119*, 2363 – 2370; c) Y. Shao, S. B. H. Kent, *Tetrahedron Lett.* **1998**, *39*, 3911 – 3914.

[15] Control experiments indicate that a common *o*-SH-phenolic ester intermediate (**I′**, Scheme 1) was generated under both AgCl and TCEP conditions. Although AgCl was known to be responsible for the slow cleavage of disulfide, its role in the subsequent activation to the HOOBt ester is under question. TCEP·HCl mediated the same S–S cleavage in a fast and quantitative fashion to generate **I′**. The role of the *o*-OH group in the activation step needs to be further established. For previous literature which may bear on mechanistic issues, see a) L. Zhang, J. Tam, *Tetrahedron Lett.* **1997**, *38*, 4375 – 4378; b) J. Verhaeghe, E. Lacassie, M. Bertrand, Y. Trudelle, *Tetrahedron Lett.* **1993**, *34*, 461 – 464; c) G. Ivanova, E. Bratovanova, D. Petkov, *J. Pept. Sci.* **2002**, *8*, 8 – 12.

[16] L. A. Carpino, D. Ionescu, A. El-Faham, *J. Org. Chem.* **1996**, *61*, 2460 – 2465.

[17] a) H. Hojo, Y. Matsumoto, Y. Nakahara, E. Ito, Y. Suzuki, M. Suzuki, A. Suzuki, Y. Nakahara, *J. Am. Chem. Soc.* **2005**, *127*, 13720 – 13725; b) E. Haginoya, H. Hojo, Y. Nakahara, Y. Nakarara, Yoshiaaki, K. Nabeshima, B. P. Toole, Y. Watanabe, *Biosci. Biotechnol. Biochem.* **2006**, *70*, 1338 – 1349.

[18] a) E. C. Johnson, S. B. H. Kent, *J. Am. Chem. Soc.* **2006**, *128*, 6640 – 6646; b) D. Bang, B. L. Pentelute, S. B. H. Kent, *Angew. Chem.* **2006**, *118*, 4089 – 4092; *Angew. Chem. Int. Ed.* **2006**, *45*, 3985 – 3988.

[19] Of course, at the present time, the total synthesis of complex glycoproteins might appear to some as a strictly academic exercise. However, we can readily envision that the required building blocks would become commercially available. At that stage, the total synthesis route could well be very useful in producing homogeneous products in amounts necessary for early stage biological evaluation.