pubs.acs.org/joc

Photoinduced Addition of Glycosyl Thiols to Alkynyl Peptides: Use of Free-Radical Thiol−Yne Coupling for Post-Translational Double-Glycosylation of Peptides

Mauro Lo Conte,† Salvatore Pacifico,† Angela Chambery,‡ Alberto Marra,† and Alessandro Dondoni*,†

†*Dipartmento di Chimica, Laboratorio di Chimica Organica, Università di Ferrara, Via L. Borsari 46, 44100 Ferrara, Italy, and* ‡*Dipartmento di Scienze della Vita, II Università di Napoli, Via Vivaldi 43, I-81100 Caserta, Italy*

adn@unife.it

Received April 26, 2010

Glu-Cys-Gly → (Br; NH_3, H_2O, r.t., 3 h) → (β-D-Glc-SH; DPAP, MeOH, hν, 45 min)

Double glycosylation of cysteine-containing peptides has been carried out by a one-pot two-step sequence comprising selective *S*-propargylation followed by photoinduced (λ_{max} 365 nm) free-radical hydrothiolation with glycosyl thiols. Conditions were established for the sequential introduction of two different thiol residues such as a glycosyl and a biotinyl derivative.

It is well established that protein glycosylation is a post-translational modification that profoundly affects protein folding, stability, immunogenicity, and biological properties and activities.[1] As native glycoproteins are isolated as mixtures of glycoforms, there is a pressing need for synthetic glycopeptides and glycoproteins with a well-defined structure and composition.[2] These compounds may serve as probes for studies in glycobiology as well as leads toward the development of pharmaceutical agents, such as carbohydrate-based anticancer vaccines.[3,4] Hence, the development of methods for peptide and protein glycosylation by efficient and site-specific ligation tools is at the forefront in biotechnology and proteomics. Synthetic glycopeptides and glycoproteins containing unnatural linkages between the carbohydrate and aglycone moieties have been reported in recent years.[5] One of the most used of these unnatural linkages is the 1,4-disubstituted triazole ring[3b,6,7] due to its robusteness[8] and ease of formation by the Huisgen Cu(I)-catalyzed azide−alkyne cycloaddition.[9] Furthermore, *S*-linked glycoproteins, i.e. compounds featuring an *S*-glycosidic bond, have been prepared by Michael addition of glycosyl thiols to synthetic dehydroalanine-containing proteins[10] and by phosphine-mediated dechalcogenation of a disulfide protein.[11] Very recently Davis and co-workers reported on free-radical addition of glycosyl thiols to genetically modified proteins in which an homoallylglycine tag was introduced.[12] Almost at the same time we reported a complementary method leading to *C*-linked glycopeptides and a glycoprotein, i.e. compounds featuring a *C*-glycosidic bond. To this end, we employed the photoinduced coupling between allyl *C*-glycosides and cysteine-containing peptides and the natural protein bovine serum albumine (BSA).[13] Both Davis and our method demonstrated how the free-radical thiol−ene coupling can be exploited as a click ligation tool for bioconjugation. Indeed, while the potential of thiol−ene reaction is amply documented in polymer and material synthesis,[14,15] its use as a metal-free ligation process in bioorganic chemistry is relatively scanty.[16] Nevertheless, the assembly of biomolecules under mild and neutral reaction conditions through the specific formation of robust sulfide bridges is an attractive target due to convenient features of the C−S bond.[17] In this context we would like to report here on the application of a sister reaction to the thiol−ene, that is the radical-mediated hydrothiolation of terminal alkyne (thiol−yne process). This reaction, whose

(1) (a) Varki, A. *Glycobiology* **1993**, *3*, 97–130. (b) Dwek, R. A. *Chem. Rev.* **1996**, *96*, 683–720. (c) Seitz, O. *ChemBioChem* **2000**, *1*, 214–246. (d) *Essentials of Glycobiology*; Varki, A., Cummings, R. D., Esko, J. D., Freeze, H. H., Hart, G. W., Marth, J., Eds.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 1999.

(2) (a) Hang, H. C.; Bertozzi, C. R. *Acc. Chem. Res.* **2001**, *34*, 727–736. (b) Davis, B. G. *Chem. Rev.* **2002**, *102*, 579–601. (c) Wong, C.-H. *J. Org. Chem.* **2005**, *70*, 4219–4225. (d) Hackenberger, C. P. R.; Schwarzer, D. *Angew. Chem., Int. Ed.* **2008**, *47*, 10030–10074. (e) Gamblin, D. P.; Scanlan, E. M.; Davis, B. G. *Chem. Rev.* **2009**, *109*, 131–163. (f) Kent, S. B. H. *Chem. Soc. Rev.* **2009**, *38*, 338–351. (g) Davis, B. G. *Angew. Chem., Int. Ed.* **2009**, *48*, 4674–4678. (h) Payne, R. J.; Wong, C.-H. *Chem. Commun.* **2010**, *46*, 21–43.

(3) (a) Danishefsky, S. J.; Allen, J. R. *Angew. Chem., Int. Ed.* **2000**, *39*, 836–863. (b) Wan, Q.; Chen, J.; Chen, G.; Danishefsky, S. J. *J. Org. Chem.* **2006**, *71*, 8244–8249.

(4) Buskas, T.; Thompson, P.; Boons, G.-J. *Chem. Commun.* **2009**, 5335–5349.

(5) (a) Specker, D.; Wittmann, V. *Top. Curr. Chem.* **2007**, *267*, 65–107. (b) Nicotra, F.; Cipolla, L.; Peri, F.; La Ferla, B.; Redaelli, C. *Adv. Carbohydr. Chem. Biochem.* **2007**, *61*, 353–398.

(6) (a) van Kasteren, S. I.; Kramer, H. B.; Jensen, H. H.; Campbell, S. J.; Kirkpatrick, J.; Oldham, N. J.; Anthony, D. C.; Davis, B. G. *Nature* **2007**, *446*, 1105–1109. (b) Gamblin, D. P.; van Kasteren, S. I.; Chalker, J. M.; Davis, B. G. *FEBS J.* **2008**, *275*, 1949–1959.

(7) Lee, D. J.; Mandal, K.; Harris, P. W. R.; Brimble, M. A.; Kent, S. B. H. *Org. Lett.* **2009**, *11*, 5270−5273 and references cited therein.

(8) Dondoni, A. *Chem. Asian J.* **2007**, *2*, 700–708.

(9) Meldal, M.; Tornøe, C. W. *Chem. Rev.* **2008**, *108*, 2952–3015.

(10) (a) Wang, J.; Schiller, S. M.; Schultz, P. G. *Angew. Chem., Int. Ed.* **2007**, *46*, 6849–6851. (b) Bernardes, G. J. L.; Calker, J. M.; Errey, J. C.; Davis, B. G. *J. Am. Chem. Soc.* **2008**, *130*, 5052–5053.

(11) Bernardes, G. J. L.; Grayson, E. J.; Thompson, S.; Chalker, J. M.; Errey, J. C.; El Oualid, F.; Claridge, T. D. W.; Davis, B. G. *Angew. Chem., Int. Ed.* **2008**, *47*, 2244–2247.

(12) Floyd, N.; Vijayakrishnan, B.; Koeppe, J. R.; Davis, B. G. *Angew. Chem., Int. Ed.* **2009**, *48*, 7798–7802.

(13) Dondoni, A.; Massi, A.; Nanni, P.; Roda, A. *Chem.—Eur. J.* **2009**, *15*, 11444–11449.

(14) (a) Lowe, A. B. *Polym. Chem.* **2010**, *1*, 17–36. (b) Kade, M. J.; Burke, D. J.; Hawker, C. J. *J. Polym. Sci., Part A: Polym. Chem.* **2010**, *48*, 743–750.

(15) Hoyle, C. E.; Bowman, C. N. *Angew. Chem., Int. Ed.* **2010**, *49*, 1540–1573.

(16) Dondoni, A. *Angew. Chem., Int. Ed.* **2008**, *47*, 8995–8997.

(17) Fiore, M.; Marra, A.; Dondoni, A. *J. Org. Chem.* **2009**, *74*, 4422–4425.

Published on Web 06/09/2010 DOI: 10.1021/jo1008178

© 2010 American Chemical Society

SCHEME 1

SCHEME 2

discovery dates back to the mid-1900s,[18] serves to introduce two thiol fragments across a carbon−carbon triple bond via a multistep mechanism as depicted in Scheme 1.

The first, slower, step involves the anti-Markovnikov-like addition of a thiyl radical to the C≡C bond to yield an intermediate vinyl thioether that is capable of undergoing a second, faster, thiyl radical addition, a formal thiol−ene reaction, leading to the dithioether with exclusive 1,2-addition mode.[19] Notably, the thiol−yne reaction can be photoinitiated in the UV−visible range (254−470 nm) and proceeds at room temperature with high efficiency in the presence of oxygen/water. Finally, the tolerance to a wide range of functional groups and orthogonality to various types of chemistry make this reaction an ideal and broadly applicable tool for multiple bioconjugation. However, while recent work highlighted thiol−yne chemistry as a valuable tool in polymer and material science,[20] its use in organic synthesis is limited to a few cases such as for example the synthesis of small vinyl sulfides and dithioether adducts.[21] Thus, the potential of this process as a chemoselective and bioorthogonal ligation tool[22] for multiple hydrothiolation of biologically relevant molecules remains to be established. Considering the above attributes, we envisioned the fabrication of dually glycosylated peptides using the photoinduced thiol−yne reaction between glycosyl thiols and alkynyl peptides. It is conceivable that the peptide double glycosylation can affect much more substantially than monoglycosylation the peptide structure and biological activity. Thus, at first a method for introducing into peptides a chemical handle

(18) Bader, H.; Cross, L. C.; Heilbron, I.; Jones, E. R. H. *J. Chem. Soc.* **1949**, 619–623.

(19) Yu, B.; Chan, J. W.; Hoyle, C. E.; Lowe, A. B. *J. Polym. Sci., Part A: Polym. Chem.* **2009**, *47*, 3544–3557.

(20) Lowe, A. B.; Hoyle, C. E.; Bowman, C. N. *J. Mater. Chem.* **2010**, *20*, 4745−4750.

(21) Benati, L.; Capella, L.; Montevecchi, P. C.; Spagnolo, P. *J. Org. Chem.* **1995**, *60*, 7941–7946.

(22) Sletten, E. M.; Bertozzi, C. R. *Angew. Chem., Int. Ed.* **2009**, *48*, 6974–6998.

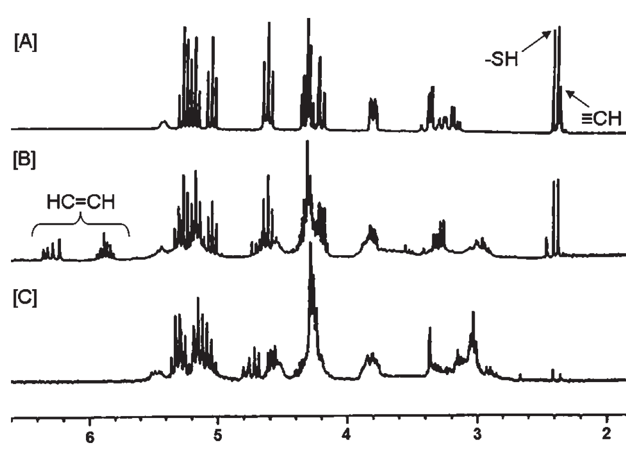

FIGURE 1. Partial ^{1}H NMR spectra of the reaction mixtures between *S*-propargyl cysteine **2** and glucosyl thiol **3** (4 equiv): initial reaction mixture (spectrum A); after 20 min irradiation at λ_{max} 365 nm (spectrum B); after 45 min irradiation at λ_{max} 365 nm (spectrum C).

bearing a propargylic tag was established by exploiting the unique reactivity of the cysteine side chain thiol.[23] As shown in Scheme 2 the model reaction of protected cysteine **1** with a slight excess of propargyl bromide (1.2 equiv) and triethylamine (2 equiv) in CH_2Cl_2 at room temperature afforded the *S*-propargyl derivative **2** in 86% yield.

Then, the photoinduced reaction of **2** with the peracetylated glucosyl thiol **3** was examined. Taking advantage of the expertise acquired from our recent work on thiol−ene coupling,[13] the reaction was carried out in methanol as the solvent by irradiation at λ_{max} 365 nm in the presence (10 mol %) of 2,2-dimethoxy-2-phenylacetophenone (DPAP) as the sensitizer. No effort was made to exclude air and moisture. After some experimentation, the use of excess thiol **3** (4 equiv) with respect to alkyne **2** was optimized. Within 45 min at room temperature, NMR analysis of the reaction mixture showed the complete consumption of starting alkyne and vinyl thioether intermediate as judged by the absence of signals at 2.26 (C≡C−H) and 5.75−6.30 ppm (HC=CH) (Figure 1). Also the doublet at 2.4 ppm corresponding to the thiol proton was not any more detectable as the excess of this reagent was transformed into the corresponding disulfide by homocoupling. The glycosylated bisthioether **4** was isolated by chromatography (53%) as a ca. 1:1 mixture of diastereomers.

The glycosylation of small peptides by the strategy outlined above was examined by using the natural tripeptide Glu-Cys-Glc (glutathione, GSH, **5**) and the synthetic tetrapeptide Arg-Gly-Asp-Cys (RGDC, **6**) as substrates. The main difference between **5** and **6** was that the former contained an internal cysteine residue while the latter was a *C*-terminal cysteine derivative. A one-pot two-step sequence comprising peptide alkynylation and thiol−yne coupling was planned in order to avoid intermediate isolation. Notably, the use of unprotected peptides GSH **5** and RGDC **6** allowed the alkynylation to be performed under very mild conditions, that is with propargyl bromide (1.1 equiv) and

(23) (a) Chalker, J. M.; Lin, Y. A.; Boutureira, O.; Davis, B. G. *Chem. Commun.* **2009**, 3714–3716. (b) Chalker, J. M.; Bernardes, G. J. L.; Lin, Y. A.; Davis, B. G. *Chem. Asian J.* **2009**, *4*, 630–640. (c) Hong, V.; Kislukhin, A. A.; Finn, M. G. *J. Am. Chem. Soc.* **2009**, *131*, 9986–9994.

TABLE 1. Synthesis of Bis-Glycosylated Peptides via One-Pot Propargylation and Photoinduced Thiol−Yne Coupling

Entry	Peptide	Sugar Thiol	Product	Yield[a] (%)
1	Glu-Cys-Gly (**5**)	β-D-Glc-SH (**9**)	**12** R^1 = H, R^2 = OH	77 (>95)[b]
2	Glu-Cys-Gly (**5**)	β-D-Gal-SH (**10**)	**13** R^1 = OH, R^2 = H	74 (>95)[b]
3	Glu-Cys-Gly (**5**)	β-D-Lac-SH (**11**)	**14**	51 (>95)[b]
4	Arg-Gly-Asp-Cys (**6**)	β-D-Glc-SH (**9**)	**15** R^1 = H, R^2 = OH	36 (>95)[b]
5	Arg-Gly-Asp-Cys (**6**)	β-D-Gal-SH (**10**)	**16** R^1 = OH, R^2 = H	27 (>95)[b]
6	Arg-Gly-Asp-Cys (**6**)	β-D-Lac-SH (**11**)	**17**	55 (>95)[b]
7	H-Asp-Leu-Tyr-Cys-Tyr-Glu-Gln-Leu-NH_2 (**7**)	β-D-Glc-SH (**9**)	**18**	(>95)[b]
8	H-Phe-Gly-Trp-Cys-Tyr-Lys-Leu-Val-OH (**8**)	β-D-Glc-SH (**9**)	**19**	(>95)[b]

[a]Isolated yields after chromatography on Sephadex LH20 column. [b]Conversion determined from the reacted alkyne as estimated by ^{1}H NMR analysis of the crude reaction mixture.

NH_3 in water at 0 °C for 1 h and then at room temperature (ca. 22 °C) for 2 h. From the NMR and MS spectra of the crude reaction mixtures the purity of the alkynyl peptides thus formed was estimated to be 80−85%. Hence, the photoinduced double hydrothiolation was carried out by using 4 equiv of the unprotected glucosyl thiol[12] **9** under the conditions outlined above with the only change being that the solvent was a 1:1 H_2O/MeOH mixture. We were pleased to observe that thiol−yne reactions took place with great efficiency with conversion >95% into the corresponding *S*-glucosyl peptides **12** and **15** as determined by ^{1}H NMR analysis of the crude reaction mixtures (Table 1). The same approach was carried out with galactosyl thiol[12] **10** and lactosyl thiol[12] **11** to give the corresponding *S*-glycopeptides **13**, **14**, **16**, and **17**. All glycopeptides **12**−**17** were isolated by chromatography on a Sephadex LH20 column as ca. 1:1 mixtures of diastereomers as established by ^{13}C NMR analysis (multiple signals for anomeric carbons at ca. δ 85 ppm). Unfortunately, attempts to separate the isomers were unsuccessful. It has to be noted that isolated yields quoted in Table 1 largely reflect difficulties handling amphiphilic products.

The scope of the one-pot two-step glycosylation strategy was broadened by using higher peptides such as the synthetic cysteine-containing octapeptides **7** and **8** (Table 1). Adopting the above procedure and conditions employed for propargylation of GSH **5** and RGDC **6** followed by thiol−yne coupling with thiol **9**, these peptides were transformed into

SCHEME 3

Glu-Cys-Gly (**5**) → propargyl bromide, NH_3, H_2O, r.t., 4 h → **9** (1.1 eq.), DPAP, MeOH, hν, 15 min → **20**, DPAP, MeOH, hν, 45 min → **21** (34%)

the corresponding *S*-glycosides **18** and **19**. In each case almost total conversion was registered by NMR analysis while chromatography over the Sephadex LH20 column allowed for isolating small samples of pure compounds for analytical purposes. That the propargylation and subsequent glycosylation involved only the peptide cysteine residue was confirmed by ESI-QTOF MS/MS analysis of **15** and **19**. The clear fragmentation pattern revealed both the *y* and *b* fragment ions, easily allowing the sequence deconvolution (see Figures S1 and S2 in the Supporting Information). Within the derived sequence, the Cys* assignment corresponded to the cysteine residue derivatized with the glycosylated thioether linker. This is noteworthy because the peptides **6** and **8** contained a basic arginine and lysine residue, respectively, and therefore there was the possibility of nonselective propargylation. Evidently this event did not occur due to the superior nucleophilicity of the cysteine sulfhydryl group with respect to the basic nitrogen atoms of other amino acid residues.

Having observed in earlier coupling experiments of alkynyl cysteine **2** with sugar thiol **3** that the use of 2−2.5 equiv of the latter afforded a considerable amount of vinyl thioether after short irradiation time, it appeared that the thiyl radical addition to the internal alkene was slow presumably due to steric considerations. In contrast to the mechanism established for the thiol−yne coupling in model systems,[19] the kinetics of the above reaction suggested the possibility to perform the sequential hydrothiolation of alkynyl peptides by two thiyl radicals generated from different thiols. The feasibility of this approach was demonstrated by using GSH **5** as the starting material. Thus, a solution of crude *S*-propargyl GSH in MeOH was treated with glucosyl thiol **9** (1.1 equiv) and sensitizer DPAP (0.1 equiv), and the mixture was irradiated at λ_{max} 365 nm for 15 min (Scheme 3). The ^{1}H NMR spectrum showed the total consumption of the alkynyl derivative. Then, a solution of biotin thiol **20** (2 equiv) in MeOH was added and the mixture was again irradiated at λ_{max} 365 nm for 45 min. The NMR spectrum of the crude reaction mixture showed the presence of vinyl proton signals at ca. 6.3 and ca. 5.6 ppm, which corresponded to 5−10% of unreacted glycosylated vinylthioether intermediate. Nevertheless, chromatography over the Sephadex LH20 column allowed the dithioether **21** to be isolated in 34% yield. The same procedure was employed for the preparation of the regioisomer of **21** with use of a reversal order of hydrothiolation, i.e. biotinylation first and then glycosylation (see the Supporting Information). As biotin is an important biological tag[24] that is typically conjugated to peptides and proteins via primary amines (e.g., lysines), this method constitutes an alternative approach that can be employed for the selective biotin labeling of cysteine-containing compounds.

In summary, we have demonstrated the selective propargylation of cysteine-containing peptides followed by coupling with glycosyl thiols as a one-pot two-step platform for post-translational dual glycosylation of peptides. Single glycosylation and conjugation with biotin also have been performed. Although demonstrated here for peptides, this approach is certainly extendable to proteins exposing a cysteine SH. As a support to this expectation are the mild reaction conditions under which the photoinduced thiol−yne coupling takes place (water as a solvent, room temperature, no metal-based catalyst) as well as irradiation at wavelengths compatible with protein stability. The lack of diastereoselectivity observed with peptides can be inconsequential when only one protein diastereomer interacts stereospecifically with enzyme or receptors.[10b] Furthermore, the relatively low abundance of natural proteins displaying cysteine residues should be compensated by the approach to proteins incorporating a single cysteine via site-synthetic mutagenesis. Research on these topics is underway in our group.

Experimental Section

Glycopeptide 12. To a cooled (0 °C), stirred solution of glutathione **5** (13 mg, 0.042 mmol) in MeOH (0.25 mL) were added NH_4OH (0.25 mL of a 28% solution in H_2O) and propargyl bromide (3.3 μL, 0.046 mmol). The solution was stirred at 0 °C for 1 h, then warmed to rt, stirred for an additional 2 h, and then concentrated. A stirred solution of the crude product, glucosyl thiol **9** (33 mg, 0.168 mmol), and DPAP (4 mg, 0.017 mmol) in 2:1 MeOH−H_2O (1.2 mL) was irradiated (λ_{max} 365 nm) at rt for 45 min and then concentrated. The residue was eluted from a column of Sephadex LH20 with MeOH to give **12** (24 mg, 77%) as an amorphous solid. ^{1}H NMR (300 MHz, D_2O) selected data: δ 4.46 (d, 1H, $J_{1,2}$ = 9.5 Hz, H-1 Glc), 3.70 (s, 2H), 2.79 (ddd, 1H, J = 5.0, 8.6, 14.2 Hz), 2.44 and 2.38 (2 ddd, 2H, J = 7.5, 7.5, 15.0 Hz), 2.03 (ddd, 2H, J = 7.0, 7.5, 7.5 Hz). ^{13}C NMR (75 MHz, D_2O): δ 175.4 (C), 174.8 (C), 173.8 (C), 171.9 (C), 86.4 (CH), 85.1 (CH), 85.0 (CH), 84.6 (CH), 79.8 (CH), 77.1 (CH), 72.4 (CH), 72.2 (CH), 69.4 (CH), 60.8 (CH_2), 54.0 (CH), 53.1 (CH), 48.8 (CH), 45.6 (CH), 44.9 (CH), 42.8 (CH_2), 36.0 (CH_2), 35.8 (CH_2), 35.2 (CH_2), 34.1 (CH_2), 33.5 (CH_2), 31.3 (CH_2), 26.1(CH_2). HRMS (ESI/Q-TOF) *m*/*z* calcd for $C_{25}H_{44}N_3O_{16}S_3$ $(M + H)^+$ 738.1884, found 738.1874.

Acknowledgment. We thank Dr. Claudio Trapella (UFPeptides s.r.l., Ferrara, Italy) for a generous gift of peptides **7** and **8**.

Supporting Information Available: Experimental procedures, physical data, ESI-QTOF MS/MS analyses, and copies of the NMR spectra of the new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

(24) (a) Chapman-Smith, A.; Cronan, J. E. *Trends Biochem. Sci.* **1999**, *24*, 359–363. (b) Yeo, D. S. Y.; Srinivasan, R.; Chen, G. Y. J.; Yao, S. Q. *Chem.—Eur. J.* **2004**, *10*, 4664–4672.