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Olefin Cross-Metathesis on Proteins: Investigation of Allylic Chalcogen Effects and Guiding Principles in Metathesis Partner Selection

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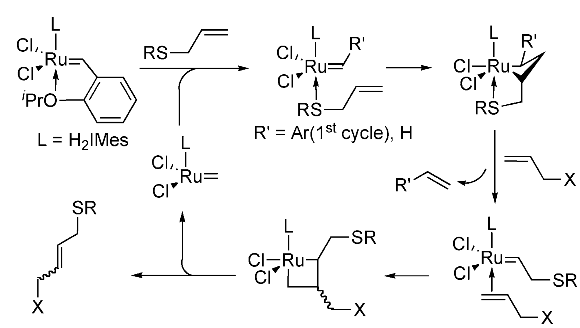
Abstract: Olefin metathesis has recently emerged as a viable reaction for chemical protein modification. The scope and limitations of olefin metathesis in bioconjugation, however, remain unclear. Herein we report an assessment of various factors that contribute to productive cross-metathesis on protein substrates. Sterics, substrate scope, and linker selection are all considered. It was discovered during this investigation that allyl chalcogenides generally enhance the rate of alkene metathesis reactions. Allyl selenides were found to be exceptionally reactive olefin metathesis substrates, enabling a broad range of protein modifications not previously possible. The principles considered in this report are important not only for expanding the repertoire of bioconjugation but also for the application of olefin metathesis in general synthetic endeavors.

Introduction

Precise modification of proteins allows the dissection and analysis of many biological systems.[1,2] While many advances in bioorthogonal ligation have been reported, there is still only a limited set of transformations that are inert to the biological milieu.[2] This privileged set of transformations must be chemoselective and efficient in aqueous media for general utility in protein modification. Olefin metathesis has emerged among a list of popular transition metal-mediated transformations as a potential candidate for selective carbon−carbon bond formation on proteins.[3] Olefin metathesis enables the installation of a carbon−carbon bond which is largely inert to a range of biological processes. Olefin cross-metathesis (CM) is also an attractive chemical challenge since aqueous olefin metathesis, while advancing,[4−6] is still in its infancy.

Scheme 1. Sulfur-Assisted Cross-Metathesis



In our exploratory work in aqueous cross-metathesis,[7] the amino acid *S*-allylcysteine (Sac) was found to be a reactive substrate in olefin metathesis. When compared to its all-carbon analogue homoallylglycine (Hag), *S*-butenyl- and *S*-pentenylcysteine, Sac was the most reactive and was the only residue to afford a synthetically useful amount of CM product.[7] Allyl sulfide reactivity in CM, much less aqueous CM, was at first counterintuitive since sulfur is known to confound many metal-mediated reactions.[8] The unique reactivity of Sac was explained with a sulfur-assisted mechanism whereby sulfur precoordination of the allyl sulfide to the ruthenium center increases the effective concentration of the alkylidene and alkene metathesis partner without detrimental chelation (Scheme 1). While allyl sulfides

(1) (a) Carrico, I. S. *Chem. Soc. Rev.* **2008**, *37*, 1423–1431. (b) Gamblin, D. P.; van Kasteren, S. I.; Chalker, J. M.; Davis, B. G. *FEBS J.* **2008**, *275*, 1949–1959. (c) Hermanson, G. T. *Bioconjugation Techniques*, 2nd ed.; Academic Press: New York, 2008. (d) Baslé, E.; Joubert, N.; Pucheault, M. *Chem. Biol.* **2010**, *17*, 213–227.
(2) (a) Hackenberger, C. P. R.; Schwarzer, D. *Angew. Chem., Int. Ed.* **2008**, *47*, 10030–10074. (b) Sletten, E.; Bertozzi, C. *Angew. Chem., Int. Ed.* **2009**, *48*, 6974–6998. (c) Lim, R. K. V.; Lin, Q. *Chem. Commun.* **2010**, 1589–1600.
(3) (a) Antos, J. M.; Francis, M. B. *Curr. Opin. Chem. Biol.* **2006**, *10*, 253–262. (b) Binder, J. B.; Raines, R. T. *Curr. Opin. Chem. Biol.* **2008**, *12*, 767–773. (c) Chalker, J. M.; Wood, C. S. C.; Davis, B. G. *J. Am. Chem. Soc.* **2009**, *131*, 16346–16347. (d) Lin, Y. A.; Chalker, J. M.; Davis, B. G. *ChemBioChem* **2009**, *10*, 959–969.
(4) (a) Hong, S. H.; Grubbs, R. H. *J. Am. Chem. Soc.* **2006**, *128*, 3508–3509. (b) Jordan, J. P.; Grubbs, R. H. *Angew. Chem., Int. Ed.* **2007**, *46*, 5152–5155.
(5) (a) Binder, J. B.; Blank, J. J.; Raines, R. T. *Org. Lett.* **2007**, *9*, 4885–4888. (b) Gułajski, Ł.; Michrowska, A.; Naroznik, J.; Kaczmarska, Z.; Rupnicki, L.; Grela, K. *ChemSusChem* **2008**, *1*, 103–109.
(6) (a) Lipshutz, B. H.; Aguinaldo, G. T.; Ghorai, S.; Voigtritter, K. *Org. Lett.* **2008**, *10*, 1325–1328. (b) Burtscher, D.; Grela, K. *Angew. Chem., Int. Ed.* **2009**, *48*, 442–454.
(7) Lin, Y. A.; Chalker, J. M.; Floyd, N.; Bernardes, G. J. L.; Davis, B. G. *J. Am. Chem. Soc.* **2008**, *130*, 9642–9643.
(8) (a) Armstrong, S. K.; Christie, B. A. *Tetrahedron Lett.* **1996**, *37*, 9373–9376. (b) Mascareñas, J. L.; Rumbo, A.; Castedo, L. *J. Org. Chem.* **1997**, *62*, 8620–8621. (c) Shon, Y.-S.; Lee, T. R. *Tetrahedron Lett.* **1997**, *38*, 1283–1286. (d) Fürstner, A.; Seidel, G.; Kindler, N. *Tetrahedron* **1999**, *55*, 8215–8230.

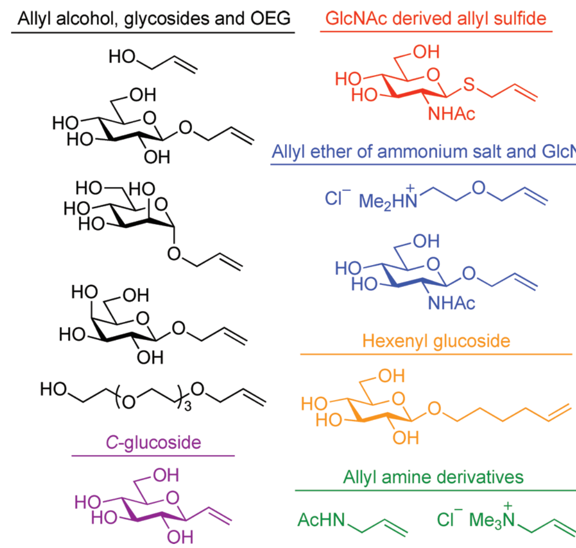
have been used in olefin metathesis previously,[9] the enhanced reactivity relative to other alkenes went unnoticed and was not exploited in synthesis. Our findings showed that allyl sulfides are not simply tolerated; they can *enhance* the rate of olefin metathesis. This enhanced rate is critical in aqueous systems where catalyst decomposition pathways may compete.[10] The reactivity of allyl sulfides in metathesis motivated the incorporation of Sac into proteins. Indeed, ready chemical access to Sac on protein surfaces enabled the first cross-metathesis on protein substrates.[7,11]

These preliminary reports demonstrated the enhanced reactivity of Sac in olefin metathesis and its use in covalent protein modification. However, the CM substrates tested were largely limited to simple allyl ethers, and the full scope of CM as a method for bioconjugation remains unclear. In fact, only a single example in these reports proceeded to completion: the cross-metathesis between the protein substrate and allyl alcohol. For general use of olefin metathesis as a bioconjugation technique, understanding the scope and limitations of metathesis substrates is essential. Indeed, Grubbs has established some guiding principles in substrate selection for CM in organic solvent,[12] yet additional factors must be considered for successful CM on protein substrates. Most notably, the reaction must proceed rapidly in water at or near room temperature. The metathesis partners and any intervening linker must therefore be selected with these stringent requirements in mind. With a clear understanding of the scope and limitations of metathesis partners, olefin metathesis may be deployed more routinely in bioconjugation. Moreover, progress in the genetic incorporation of alkene-containing unnatural amino acids further motivates the development of olefin metathesis as a method for protein modification.[13,14] Finally, an increased understanding of chemical behavior in aqueous olefin metathesis is useful in general synthetic endeavors.[15] These considerations motivate our investigation of aqueous CM on protein substrates. Herein, we report our investigation into allylic chalcogen activation effects in olefin metathesis and the scope of CM partners useful in protein cross-metathesis.

Results and Discussion

Assessing the Substrate Scope of Protein Cross-Metathesis at *S*-Allylcysteine. The model protein used for our studies was a single cysteine mutant of subtilisin from *Bacillus lentus* (SBL-S156C). Sac was installed on SBL-S156C by direct allylation with allyl chloride, in accordance to our previous report.[11] While we have previously disclosed several examples of CM on the single Sac mutant of SBL (**2**, SBL-156Sac) using Hoveyda–Grubbs second generation catalyst (**1**),[16] the substrate scope of the reaction was not fully assessed. Examples to date include simple allylic alcohols and ethers.[7,11] Since we are interested in biorelevant protein modifications, allyl ethers containing carbohydrates, oligo(ethylene glycols), and charged groups were among the metathesis substrates synthesized for this study. Compounds containing an allyl sulfide, *N*-allyl amines, or longer alkene tethers are also metathesis partners of interest for the assessment of substrate scope (Scheme 2).

Scheme 2. Alkene Substrates Used in This Investigation

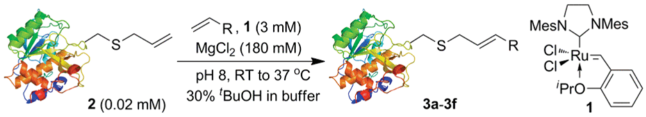


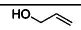
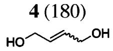
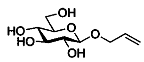
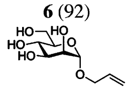
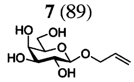
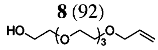
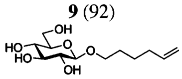
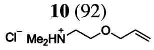
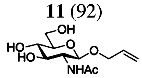
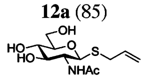
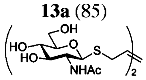
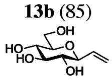
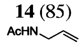
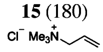
Reactions were monitored for up to 2 h, the reaction time necessary for complete conversion with allyl alcohol as the metathesis partner (Table 1, entry 1). Reaction conversions were determined by ESI-MS.[17] When protein **2** was tested with each of the substrates in CM, the best results were obtained with allylic alcohols, ethers, and hexenyl glucoside **10** (Table 1, entries 1–7). The reaction worked moderately well with allyl glycosides **6**–**8** and oligo(ethylene glycol) derivative **9**, with conversions ranging from 30 to 65%. CM with hexenyl glucoside **10** importantly revealed the sensitivity to linker length, with full conversion to the modified protein after only 1 h at room temperature (Table 1, entry 7). This result compares favorably to the allyl glycosides in entries 3–5 and was the first carbohydrate-bearing substrate to proceed with full conversion.

As an additional guide to mechanism, CM with self-metathesis product of allyl alcohol (**5**) was carried out to test whether and under what conditions it is a reactive substrate. The self-metathesis product of allyl alcohol used in Table 1 was largely the *E* isomer isolated from a model cross-metathesis in water (see ESI). Cross-metathesis of **5** with protein **2** only reached 28% conversion under the same reaction conditions as allyl alcohol (Table 1, entry 2). This result suggests that the CM in entry 1 is mainly with allyl alcohol and not with **5**. The difference in conversion is likely due to a higher rate of metathesis of **4** than **5** since the latter is a more substituted alkene and therefore generally slower in olefin metathesis.[18] However, when heated to 37 °C, the reaction with **5** proceeded

(9) (a) Spagnol, G.; Heck, M.-P.; Nolan, S. P.; Mioskowski, C. *Org. Lett.* **2002**, *4*, 1767–1770. (b) Toste, F. D.; Chatterjee, A. K.; Grubbs, R. H. *Pure Appl. Chem.* **2002**, *74*, 7–10.
(10) (a) Dinger, M. B.; Mol, J. C. *Organometallics* **2003**, *22*, 1089–1095. (b) Dinger, M. B.; Mol, J. C. *Eur. J. Inorg. Chem.* **2003**, 2827–2833.
(11) Chalker, J. M.; Lin, Y. A.; Boutureira, O.; Davis, B. G. *Chem. Commun.* **2009**, 3714–3716.
(12) Chatterjee, A. K.; Choi, T.-L.; Sanders, D. P.; Grubbs, R. H. *J. Am. Chem. Soc.* **2003**, *125*, 11360–11370.
(13) (a) van Hest, J. C. M.; Tirrell, D. A. *FEBS Lett.* **1998**, *428*, 68–70. (b) Zhang, Z.; Wang, L.; Brock, A.; Schultz, P. G. *Angew. Chem., Int. Ed.* **2002**, *41*, 2840–2842.
(14) Ai, H.-w.; Shen, W.; Brustad, E.; Schultz, P. G. *Angew. Chem., Int. Ed.* **2010**, *49*, 935–937.
(15) (a) Fürstner, A. *Angew. Chem., Int. Ed.* **2000**, *39*, 3012–3043. (b) Nicolaou, K. C.; Bulger, P. G.; Sarlah, D. *Angew. Chem., Int. Ed.* **2005**, *44*, 4490–4527. (c) Hoveyda, A. H.; Zhugralin, A. R. *Nature* **2007**, *450*, 243–251.
(16) Garber, S. B.; Kingsbury, J. S.; Gray, B. L.; Hoveyda, A. H. *J. Am. Chem. Soc.* **2000**, *122*, 8168–8179.
(17) For a discussion and validation of ESI-MS as a tool for monitoring reaction conversion on protein substrates, see Supporting Information.
(18) Kirkland, T. A.; Grubbs, R. H. *J. Org. Chem.* **1997**, *62*, 7310–7318.

Table 1. Substrate Scope of Cross-Metathesis with SBL-156Sac



Entry	Alkene (mM)	Conditions	Prod.	Conv. (%)[a]
1	 **4** (180)	RT, 2 h	**3a**	>95
2	 **5** (180)	RT, 2 h	**3a**	28
		then 37 °C, 30 mins	**3a**	>95
3	 **6** (92)	37 °C, 1 h	**3b**	30
4	 **7** (89)	37 °C, 1 h	**3c**	30
5	 **8** (92)	37 °C, 1 h	**3d**	30
6[b]	 **9** (92)	37 °C, 30 mins	**3e**	65
7	 **10** (92)	RT, 1 h	**3f**	>95
8	 **11** (92)	37 °C, 1 h	-	0
9	 **12a** (85)	37 °C, 2 h	-	0
10	 **13a** (85)	37 °C, 1 h	-	0
11	 **13b** (85)	37 °C, 1 h	-	0
12	 **14** (85)	37 °C, 1 h	-	0
13	 **15** (180)	37 °C, 1 h	-	0
14	 **16** (170)	37 °C, 1 h	-	0

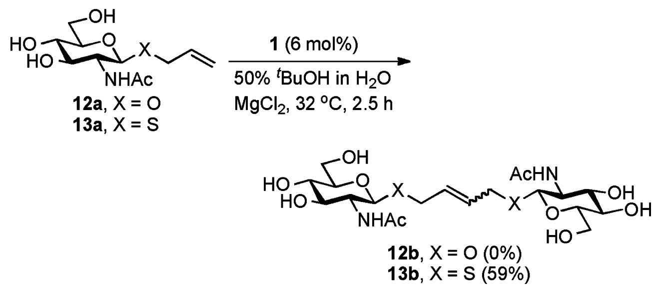
[a] Determined by LC-MS. [b] First 2 h at RT.

with over 95% conversion after 30 min. This result indicates that while slower than allyl alcohol, the self-metathesis partner can successfully re-enter the metathesis cycle (Table 1, entry 2). This observation is consistent with other reports on the reversible nature of olefin CM.[19]

A number of CM partners failed to react with Sac-containing proteins. No product formation was observed in the CM between protein **2** and substrates **11**−**16** (Table 1, entries 8−14). In all of these attempts only starting material was detected on LC-MS. These results revealed structural features of metathesis partners that may adversely affect the rate of reaction. For instance, alkenes **11** and **16** contain electron-withdrawing ammonium groups known to retard olefin metathesis.[4] *N*-Acetylglucosamine-derived substrates **12a** and **13a** also gave no detectable CM product. Comparison of GlcNAc derivative **12a** (Table 1, entry 9) with glucose derivative **6** (Table 1, entry

(19) (a) Fürstner, A.; Thiel, O. R.; Ackermann, L. *Org. Lett.* **2001**, *3*, 449–451. (b) Smith, A. B., III; Adams, C. M.; Kozmin, S. A. *J. Am. Chem. Soc.* **2001**, *123*, 990–991.

Scheme 3. Comparison in Rate of Self-Metathesis between **12a** and **13a**



3) reveals that the acetamide at C-2 in **12a** must contribute some adverse steric, electronic, or chelating influence that impedes metathesis. Allyl sulfide **13a** was synthesized with the intention of overcoming these obstacles, since this linker might be expected to be more reactive. However, only self-metathesis was observed (see ESI). To further investigate this observation, a model reaction comparing the rate of self-metathesis between **12a** and **13a** was carried out (Scheme 3). The formation of the self-metathesis product of **13a** was observed within the first 30 min of reaction. After 2.5 h, the self-metathesis product **13b** was isolated in a yield of 59%. In contrast, the self-metathesis of **12a** only resulted in >95% recovery of starting material under the same reaction conditions. When **13b** was tested in CM with protein **2**, no reaction was observed (Table 1, entry 11). Apparently, the self-metathesis product **13b**, unlike **5**, cannot re-enter the metathesis cycle. We attribute this low reactivity of **13b** to its hindered structure. The propensity for self-metathesis and the resulting reactivity of the self-metathesis product are therefore important considerations in substrate selection. Reaction with vinyl *C*-glucoside **14** was unfruitful, likely because of the steric congestion at the alkene. Allyl acetamide **15** performed poorly in CM with protein **2**, possibly due to the formation of a stable six-member ring chelate via carbonyl oxygen coordination to ruthenium, poisoning the catalyst.

From these initial results in Table 1, it seems that in order for CM on Sac-containing proteins to work efficiently, the metathesis partner needs to be slightly less reactive than the allyl sulfide. If the metathesis partner is highly reactive (e.g., **4** or **13a**), the product of self-metathesis must be able to re-enter the catalytic cycle or no protein modification is observed. Of the substrates tested, allylic alcohols and ethers, and hexenyl glucoside **10** stood out as the most productive metathesis partners for Sac-containing protein **2**. The remaining substrates (Table 1, entries 8−14) provide a benchmark of challenging transformations that can perhaps be achieved by altering the protein metathesis partner or linker. Accordingly, we turned next to an assessment of the accessibility of the Sac residue on the protein and its effect on cross-metathesis.

Making the Reactive Site More Accessible: Linker-Extended *S*-Allylcysteine on Protein Substrates. CM failed to reach full conversion with metathesis partners such as allyl glycosides and oligo(ethylene glycols). In other cases we saw no productive CM, particularly for sterically demanding and electron-poor substrates. According to the proposed sulfur-assisted metathesis mechanism, coordination of the Sac residue to the ruthenium is critical. We therefore investigated the influence of sterics at the protein surface. Accordingly, acrylamide **17** was used to install a Sac residue at the same protein site, but extended from the protein surface to create a less hindered protein olefin. Conjugate addition of the cysteinyl residue on SBL-S156C to

Scheme 4. Installing Extended *S*-Allylcysteine on Protein Surface

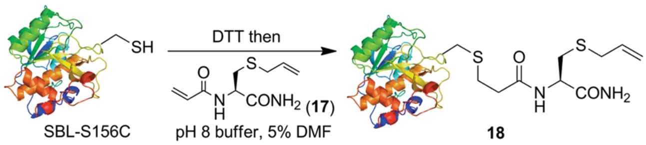
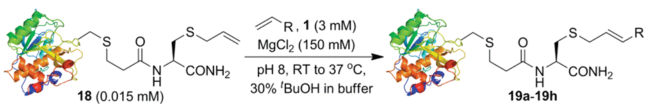
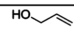
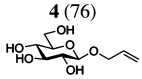
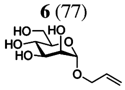
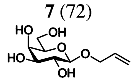
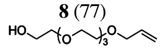
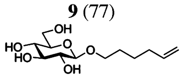
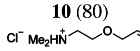
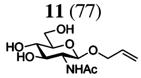
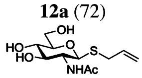
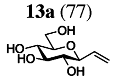
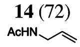
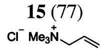


Table 2. Cross-Metathesis on SBL **18**−Extended Sac



Entry	Alkene (mM)	Conditions	Prod.	Conv. (%)[a]
1	 **4** (76)	RT, 30 mins	**19a**	>95
2	 **6** (77)	37 °C, 1 h	**19b**	>95
3	 **7** (72)	37 °C, 1 h	**19c**	>95
4	 **8** (77)	37 °C, 1 h	**19d**	>95
5	 **9** (77)	RT, 2 h	**19e**	>95
6	 **10** (80)	RT, 30 mins	**19f**	>95
7	 **11** (77)	37 °C, 30 mins	**19g**	29
8	 **12a** (72)	37 °C, 2 h	**19h**	53
9	 **13a** (77)	37 °C, 1 h	-	0
10	 **14** (72)	37 °C, 1 h	-	0
11	 **15** (77)	37 °C, 30 mins	-	0
12	 **16** (74)	37 °C, 1 h	-	0

[a] Determined by LC-MS.

acrylamide **17** led to full conversion of the alkylated protein **18** after incubation at 37 °C for 1 h (Scheme 4). The reaction at cysteine was verified with Ellman's assay (see ESI).

With a protein containing a linker-extended Sac in hand, CM with allyl alcohol (**4**) and ether substrates **6** to **12a** were carried out. Notably, all reactions proceeded to full conversions with the exception of ethanolamine **11** and GlcNAc **12a**, which gave 29 and 53% conversion, respectively (Table 2). Protein modification via CM with compound **11** and **12a**, though only achieved with low conversions, was promising because these were the only two allyl ether substrates that had failed to work with protein **2**. The results summarized in Table 2 demonstrated that steric effects are indeed important for CM on the surface of SBL. CM with allyl alcohol reached full conversion after just 30 min of reaction time at room temperature, while the same reaction with protein **2** required 2 h to proceed to completion. Protein CM with hexenyl glucoside **10** also proceeded with full conversion. Again, shorter reaction time was required with the protein containing the extended linker.

The influence of steric effects observed on our model system should be considered when a modification is desired at an active site or hindered helix. Yet steric hindrance is only one dominant factor. Substrates **13a**−**16** remained challenging, even for the Sac-extended protein **18**. Substrate **13a** again only resulted in unproductive self-metathesis (see ESI), whereas compounds **14**−**16** are either too electron-deficient or sterically demanding to participate in CM.

***Se*-Allylselenocysteine: A Metathesis Substrate Superior to *S*-Allylcysteine in Aqueous Cross-Metathesis.** From our initial report on allyl sulfides[7] and the results above, it was increasingly apparent that allylic heteroatoms modulate the rate of olefin metathesis. When considering these observations alongside reports of the positive influence of allylic alcohols[20] and ethers[14] in olefin metathesis, it is tempting to consider if this enhanced reactivity was general for allylic chalcogenides. Pursuing this hypothesis, we next examined the CM reactivity of allyl selenides. Accordingly, *Se*-allylselenocysteine (Seac) derivative **21a** was synthesized and then tested along with Sac derivative **20a** in model aqueous CM with allyl alcohol under identical reaction conditions. Indeed, the reaction with Seac **21a** was higher yielding than the Sac case, with respective yields of 72 and 56% (Scheme 5a). The difference in reactivity may be attributed to the softness of selenium which makes the coordination to ruthenium even more favorable than the sulfur in Sac. Remarkably, there are few examples in the literature describing olefin metathesis with selenium-containing compounds. In one instance, Koketsu and co-workers used RCM of an allyl selenide derivative as a key step for the synthesis of selenium-containing bicyclic β-lactams.[21] However, the scope of olefinic selenoethers in olefin metathesis was not manifested in these reports, and the enhanced reactivity of allyl selenides was not noted.

Next, a more complex and biochemically important carbohydrate metathesis partner **22** was used in place of allyl alcohol. The CM reaction of sugar **22** with Sac **20a** and Seac **21a** gave moderate yields of 45 and 53%, respectively. Initial inspection of the yields of CM products **20d** and **21d** suggests no difference in reactivity between Sac and Seac. However, self-metathesis product **21c** was also isolated from the reaction of Seac, whereas no self-metathesis product of Sac (**20c**) was observed (Scheme 5b). Total CM yields (CM and self-metathesis) are therefore 73% for Seac and 45% for Sac, a clear indication that not only are allyl selenides reactive in cross-metathesis but also they are more reactive than allyl sulfides. We sought to take advantage of this reactivty of allyl selenides in protein conjugation. In particular, the more reactive allyl selenide was tested for its ability to promote challenging CM with substrates that were sluggish or unreactive with Sac.

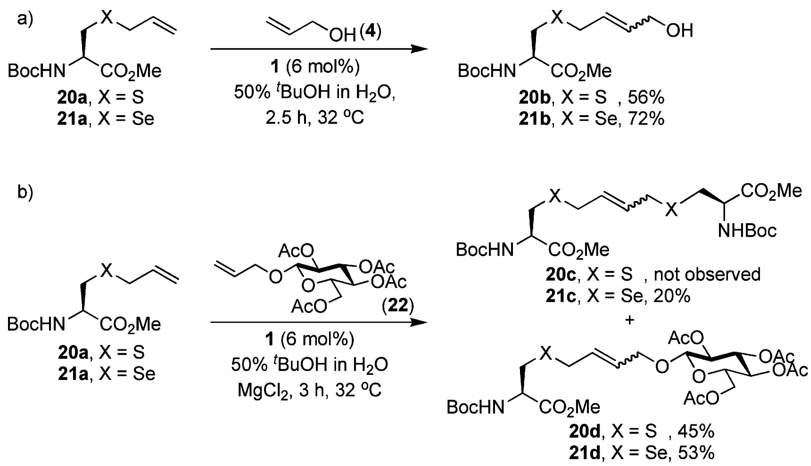
Chemical Access to *Se*-Allylselenocysteine on a Protein Surface. To directly compare the CM reactivity with protein **18**, Seac-containing protein **24** was synthesized in a similar manner using Seac acrylamide **23** (Scheme 6). Again, reaction at cysteine was verified with Ellman's assay (see ESI).

The remarkable reactivity of allyl selenide-containing protein **24** was clear after the first test for CM activity. CM between allyl alcohol and protein **24** required only *15 min at room*

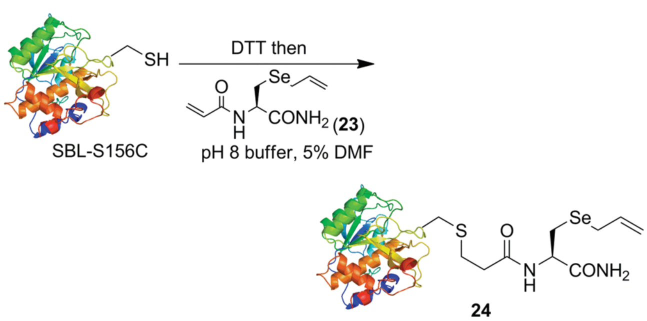
(20) (a) Hoye, T. R.; Zhao, H. *Org. Lett.* **1999**, *1*, 1123–1125. (b) Hoveyda, A. H.; Lombardi, P. J.; O'Brien, R. V.; Zhugralin, A. R. *J. Am. Chem. Soc.* **2009**, *131*, 8378–8379.

(21) Garud, D. R.; Garud, D. D.; Koketsu, M. *Org. Biomol. Chem.* **2009**, *7*, 2591–2598.

Scheme 5. Comparison of Reactivity between *S*-Allylcysteine and *Se*-Allylselenocysteine in Aqueous Cross-Metathesis

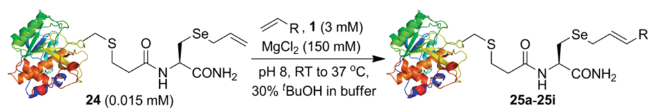


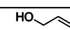
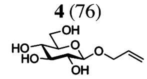
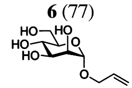
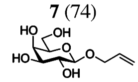
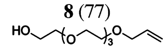
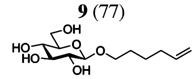
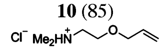
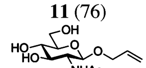
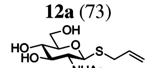
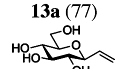
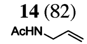
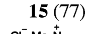
Scheme 6. Installing Extended *Se*-Allylselenocysteine on Protein Surface



temperature to reach completion (Table 3, entry 1). The LC-MS data of this reaction is shown in Scheme 7. The total ion chromatogram (TIC) was typically analyzed between 13 and 16 min, the time of elution for all protein material, both unmodified and modified. Ethers also reached full conversion under mild reaction conditions (Table 3, entries 2−8). Notably, these substrates included the more challenging ethanolamine **11** and GlcNAc **12a**, which resulted in poor conversions in previous attempts with protein **18**. CM with the reactive hexenyl glucoside **10** also gave full conversion (Table 3, entry 6). Moreover, allyl acetamide **15**, a substrate that was unreactive in all previous CM reactions, also gave productive CM with protein **24** (Table 3, entry 11). Either the unhindered Seac on protein **24** was able to initiate rapid CM with **15** before the catalyst was sequestered by the acetamide or the allyl selenide is simply a better ligand for ruthenium than the chelating acetamide. Among the CM reactions carried out on protein **24**, the modifications with GlcNAc **12a** and acetamide **15** are particularly biologically relevant modifications. The GlcNAc moiety on the glycosylated protein **25h** is an anchor for many modifications and bioprocesses such as celluar signaling.[22] Moreover, acetylation such as that found in **25i** is an important protein posttranslational modification (PTM) and often occurs either at the N-terminus or at lysine residues of proteins.[23] The attachment of an *N*-acetyl group on proteins by CM might suffice as a mimic for the natural PTM of lysine residues in proteins.[24] The results in Table 3 are highly promising, and it is clear that unhindered allyl selenides allow unprecedented reactivity in bioconjugation by olefin metathesis. Nonetheless, some limitations for CM using **24** remain. Substrates **13a**, **14**, and **16** did not participate in productive CM. Likely *C*-vinyl glucoside **14** is too hindered and **16** is both too hindered and

Table 3. Cross-Metathesis on SBL **24**−Extended Seac

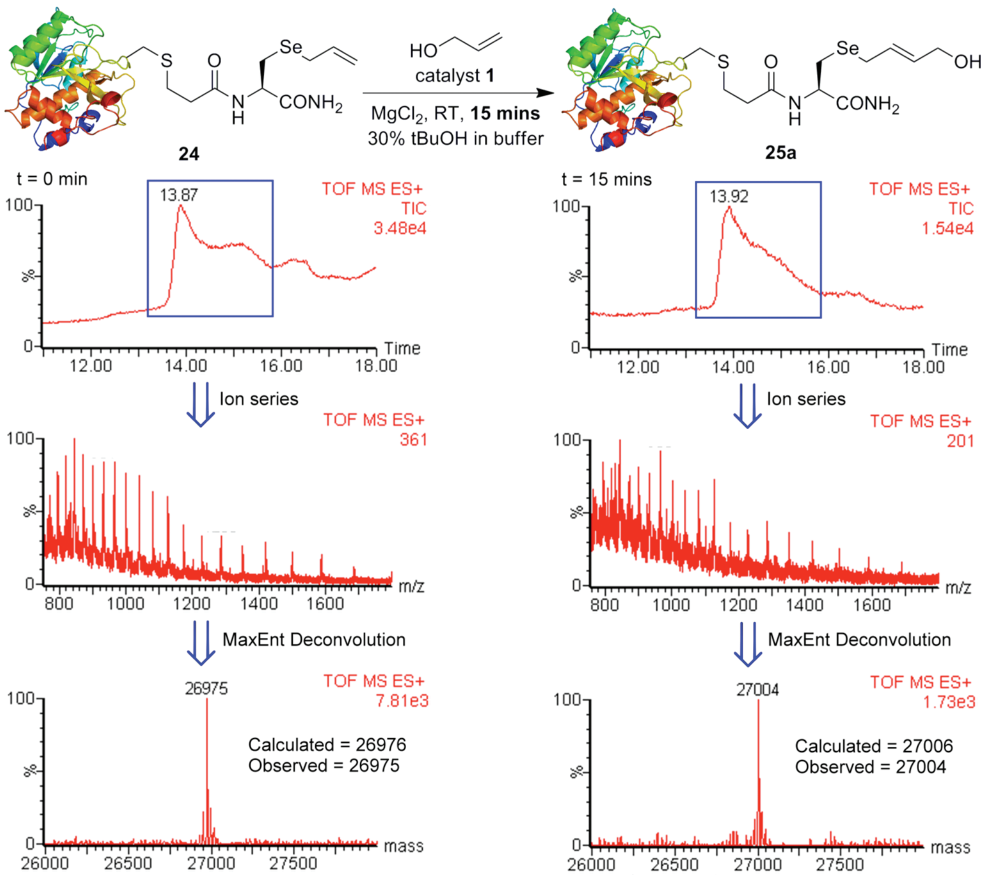


Entry	Alkene (mM)	Conditions	Prod.	Conv. (%)^a
1	 **4** (76)	RT, 15 mins	**25a**	>95
2	 **6** (77)	37 °C, 1 h	**25b**	>95
3	 **7** (74)	37 °C, 1 h	**25c**	>95
4	 **8** (77)	37 °C, 1 h	**25d**	>95
5	 **9** (77)	RT, 1 h	**25e**	>95
6	 **10** (85)	RT, 30 mins	**25f**	>95
7	 **11** (76)	37 °C, 30 mins	**25g**	>95
8	 **12a** (73)	37 °C, 1 h	**25h**	>95
9	 **13a** (77)	RT, 1 h	-	0
10	 **14** (82)	37 °C, 1 h	-	0
11	 **15** (77)	37 °C, 30 mins	**25i**	90
12	 **16** (74)	37 °C, 1 h	-	0

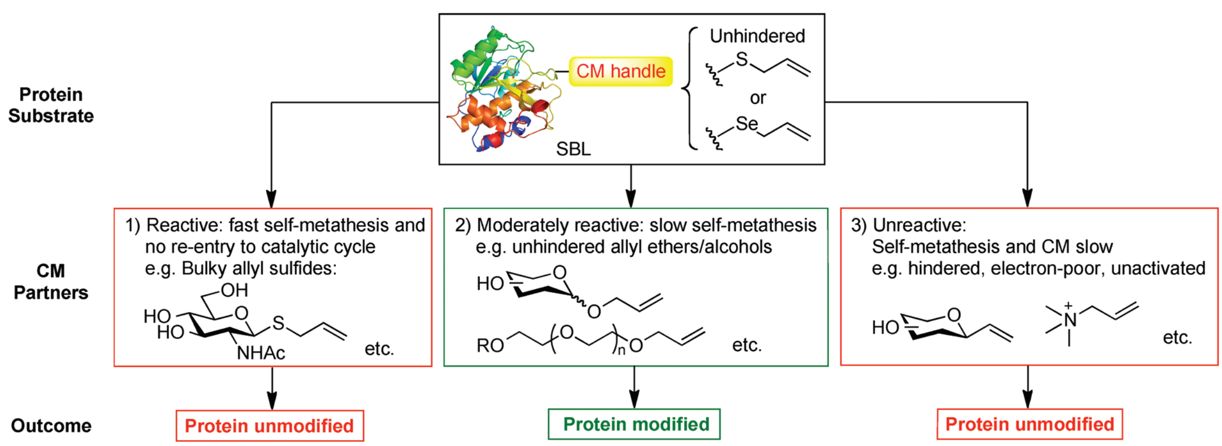
^{a} Determined by LC-MS.

(22) Hart, G. W.; Akimoto, Y. In *Essentials of Glycobiology*; 2nd ed.; Cold Spring Harbor Laboratory Press: New York, 2009; pp 263−279.
(23) Walsh, C. T.; Garneau-Tsodikova, S.; Gatto, G. J., Jr. *Angew. Chem., Int. Ed.* **2005**, *44*, 7342–7372.
(24) Davis, B. G. *Science* **2004**, *303*, 480–482.

Scheme 7. Cross-Metathesis between SBL **24** and Allyl Alcohol



Scheme 8. Summary of Substrate and Linker Selection from Cross-Metathesis on Model Protein SBL



electron deficient. Again, **13a** underwent preferential self-metathesis, and no CM product was detected.

Conclusions

In summary, by examining a range of sterically and electronically diverse olefin substrates in protein CM, we have gleaned some guiding principles for successful CM on model protein substrates (Scheme 8). In general, allyl sulfides or allyl selenides extended from the protein surface at the site of modification are desirable. The role of this olefin partner is distinct since there is minimal risk of protein self-metathesis.[25] We suspect that extending the site of the reaction from the surface of the protein enhances reactivity simply through steric relief and increased solvent accessibility. However, we cannot rule out other subtle changes in the complex chemical environment of the protein that may account for this difference in reactivity. For Sac- and Seac-containing proteins, allyl ethers make good metathesis partners because they undergo slow self-metathesis compared to allyl sulfides, allowing sufficient amount of unsubstituted alkene for productive CM. However, allyl ethers are not the only effective metathesis partners as other olefins such as hexenyl glucoside **10** can also be reactive in CM. Importantly, the metathesis partner must not form a stable chelate. If this occurs, the metathesis rate drops and little or no

(25) No protein self-metathesis was observed by SDS-PAGE gel analysis (see ESI).

protein modification is observed. Ideally, for fast protein CM the metathesis partner should also have an unhindered and nonchelating alkene tether. These results are summarized in Scheme 8.

Throughout the course of our investigation, we have demonstrated that by relieving steric hindrance around the alkene and protein surface, the rate of cross-metathesis is increased. However, the steric-sensitive nature of olefin metathesis also means that modification at more hindered protein sites is a current, unmet challenge with conventional metathesis catalysts. This limitation prompts the need for a new class of metathesis catalysts for bioconjugation, where the ligand binding to the metal should be both small and water-soluble—a significant challenge given that the sterically encumbered NHC ligands impart the stability necessary for use in air and water. Additionally, allyl selenides were discovered to be superior to allyl sulfides in aqueous CM. For unhindered allyl selenide-containing proteins, efficient CM was achieved with several substrates including carbohydrates, oligo(ethylene glycols), allyl acetamides, and even alkenes with electron-withdrawing ammonium salts. It is also worth noting that, at the time of our first report,[7] examples of homogeneous cross-metathesis in water were largely limited to simple alkenols.[4,5] In this report, a new benchmark in substrate complexity is set for olefin cross-metathesis. Complex macromolecules and metathesis partners were joined efficiently by virtue of the innate affinity of allyl sulfides and allyl selenides for ruthenium, an affinity that orchestrates rapid, productive metathesis of the alkene and alkylidene.

The promising results from CM of allyl selenides are driving our effort in developing chemical and genetic strategies for incorporation of Seac and other allyl selenide derivatives on protein surfaces. We are also investigating further the directing effect of chalcogens in olefin metathesis. We anticipate the application of these concepts and techniques in bioconjugation, and synthetic chemistry will allow a largely untapped potential of allyl sulfides and allyl selenides to be realized.

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Supporting Information Available: Full experimental procedures, including ^{1}H and ^{13}C NMR spectra for all novel compounds and ESI-MS for all protein samples. This material is available free of charge via the Internet at http://pubs.acs.org.

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