J|A|C|S
COMMUNICATIONS

Published on Web 01/04/2006

Tyrosine-Selective Protein Alkylation Using π-Allylpalladium Complexes

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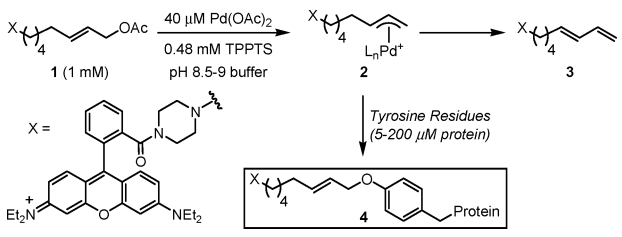
Transition metal mediated reactions are emerging as a promising set of strategies for protein modification, as organometallic intermediates can functionalize amino acid side chains that are difficult to target using traditional bioconjugation techniques. Several studies have now demonstrated that these reactions can modify proteins in aqueous solution with high chemoselectivity and virtually complete functional group tolerance.[1] Due to their versatility in organic synthesis, palladium-catalyzed reactions are a welcome addition to this list, and pioneering studies have demonstrated that palladium-catalyzed cross-coupling reactions can indeed modify aryl halides[2a,b] and boronic acids[2c] introduced into synthetic peptides.

For the direct modification of native proteins, however, we were drawn to electrophilic π-allyl complexes because these species are more appropriately matched to the nucleophilic functional groups of the natural amino acids. π-Allyl species have enjoyed widespread success in organic synthesis[3] and offer the advantage that the allylic acetate, carbonate, and carbamate precursors are completely inert until activated by the palladium catalyst. Perhaps the most relevant precedent for protein modification is the allylation of phenolic groups,[4] suggesting that tyrosine residues could be functionalized if biomolecule-compatible reaction conditions could be realized. We have found that this can be achieved, resulting in a highly selective alkylation reaction that can install allylic functionality on proteins under mild reaction conditions (Scheme 1). We report herein the conditions and selectivity of this new bioconjugation reaction, as well as a demonstration of its use for the installation of hydrophobic groups through a solubility-switching technique.

These studies began with the development of aqueous reaction conditions[5] that would be expected to preserve the tertiary structure of protein substrates. Chymotrypsinogen A (**4a**) was chosen as a model protein for these studies, as previous Mannich-type alkylation reactions have revealed the presence of a solvent-accessible tyrosine residue on the protein surface.[6] Upon exposure of a 200 μM solution of this protein to rhodamine-labeled allylic acetate **1** (1 mM), $Pd(OAc)_2$ (40 μM, 4.3 ppm Pd), and triphenylphosphine tris-(sulfonate) (TPPTS, 0.48 mM) as a water-soluble phosphine ligand, efficient protein labeling was observed within 45 min at room temperature. Analysis of the reaction mixture by ESI−MS and RP−HPLC consistently indicated 50−65% conversion of the starting protein to a singly alkylated product, corresponding to the addition of **1** after loss of the acetate group (Figure 1a).[7] A small amount of doubly alkylated product was also observed. Consistent with a mechanism involving the phenolate anion, the reaction was found to proceed best at pH 8.5−9.0.[8] Through HPLC analysis and comparison to authentic material, it was determined that excess **1** was converted to diene **3**,[9] which could easily be removed from the protein solution by gel filtration or extraction.

Scheme 1



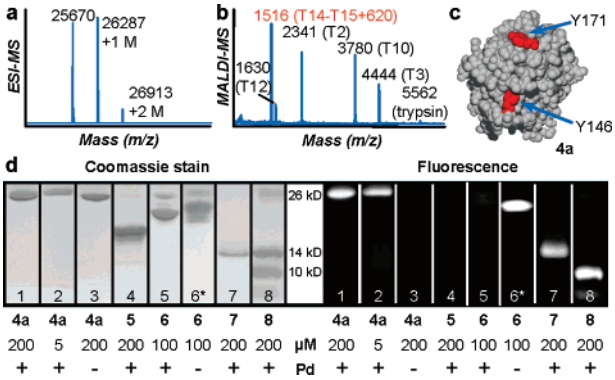


Figure 1. Alkylation of tyrosine residues with fluorescent allyl acetate **1**. (a) ESI−MS analysis of modified chymotrypsinogen A (**4a**, 200 μM). Reaction conditions: 1 mM **1**, 40 μM $Pd(OAc)_2$, 0.48 mM TPPTS, 100 mM phosphate buffer, pH 8.5, 45 min, room temperature. (b) The tyrosine selectivity of the reaction was confirmed using a trypsin digest. The modified T14−T15 fragment (containing Y171) can be identified at *m*/*z* 1516. (c) Structure of chymotrypsinogen A, indicating the location of Y171. Y146 is presumed to be the second modification site. (d) Detection of protein modification using SDS−PAGE and Coomassie staining (left) and fluorescence visualization (right). Proteins: (**5**) horse heart myoglobin, (**6**) H-Ras, (**7**) bacteriophage MS2, (**8**) α-chymotrypsin A. The modification of **7** was carried out using 200 μM $Pd(OAc)_2$; otherwise, all reactions were run as described in (a) using the indicated protein concentrations. The reaction analyzed in lane 6* was run with Oregon Green maleimide to verify the presence of a reactive cysteine residue on **6**.

The site selectivity of the reaction was determined through proteolytic digestion of the modified protein with trypsin. The T14−15 fragment, which contains Y171, was identified as the predominant site of modification (Figure 1b,c). Fragments corresponding to the alkylation of other nucleophilic side chains have not been identified to date. The second modification site is presumed to be Y146, a second surface-accessible residue. The two other tyrosine residues in the protein sequence are significantly less accessible to the reaction solvent and are presumed not to participate.

Due to the fluorescent nature of **1**, the alkylated products could also be detected using a gel-based assay. As shown in Figure 1d, similar modification levels were obtained for reactions carried out using only 5 μM **4a** (compare lanes 1 and 2). As would be expected, no labeling was detected in the absence of $Pd(OAc)_2$ (lane 3). In the presence of active catalyst, no modification was observed for horse heart myoglobin (**5**, lane 4), a protein with 19 lysines but with no surface-accessible tyrosine residues. Similarly, the reactive cysteine thiolate of H−Ras (**6**) was not modified under the reaction conditions (lane 5). The ability of this site to participate in alkylation

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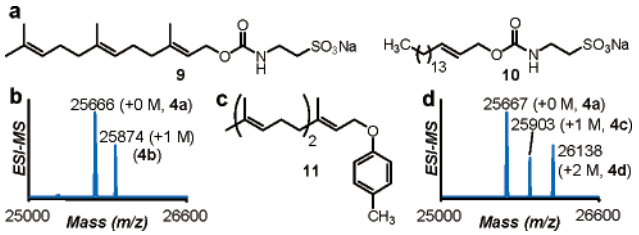

Figure 2. Preparation of synthetic lipoproteins. (a) Allylic taurine carbamates **9** and **10** serve as convenient water-soluble lipid precursors. (b) ESI−MS analysis of farnesylated **4a**. Conditions: 200 μM **4a**, 1 mM **9**, 400 μM $Pd(OAc)_2$, 2 mM TPPTS, phosphate buffer, pH 9, 100 min, room temperature. (c) To confirm the product structure, cresol adduct **11** was prepared under analogous reaction conditions. (d) Modification of **4a** using **10**. Identical reactions conditions were used, with the addition of 5% DMSO.

reactions was verified by exposing this protein to Oregon Green maleimide, a well-known reagent for the modification of cysteine residues (lane 6*). In contrast, the protein coat of bacteriophage MS2 (**7**, lane 7, demonstrated previously to possess reactive tyrosine residues[10]) was successfully modified. Finally, the reaction also proceeded to high levels of conversion when carried out on activated α-chymotrypsin A (**8**). After disulfide reduction with DTT (which cleaves the protein into three separate peptide chains), SDS−PAGE analysis indicated that only the fragment at 10 kD (corresponding to Y171) had been modified (lane 8).

A particularly attractive feature of this reaction is its ability to cleave a "disposable" group upon formation of the π-allyl complex. This can allow the aqueous solubilization of otherwise prohibitively hydrophobic molecules by coupling them to charged carrier groups, such as taurine (Figure 2a). To demonstrate this possibility, water-soluble farnesyl carbamate **9** (1 mM) was exposed to 200 μM **4a**, 2 mM TPPTS, and 400 μM $Pd(OAc)_2$ in phosphate buffer at pH 9. ESI−MS analysis indicated that 30% overall conversion to farnesylated product **4b** was observed. Although Pd-catalyzed rearrangements of the double bonds can be envisioned during this reaction, linear allyl ether **11** was the only product observed upon exposure of *p*-cresol to comparable reaction conditions.[11]

Similar success was achieved for the alkylation of **4a** using exceptionally hydrophobic C_{17} chains. Taurine carbamate **10** displays good solubility in 95:5 H_2O:DMSO mixtures and affords singly and doubly modified protein conjugates (Figure 2d). The sudden increase in doubly modified product may arise from the association of singly modified protein with additional molecules of carbamate **10** or through relaxation of the protein structure by the small amount of organic cosolvent.

This "solubility switching" strategy provides convenient access to proteins that can be incorporated into lipid membranes. To explore this possibility, a mixture of **4a**, **4c**, and **4d** (resulting from the modification of **4a** with **10**) was exposed to 100 nm small unilamellar vesicles (SUVs) for 20 min (Figure 3a). A nonionic surfactant, β-octyl-D-glucopyranoside, was also added to the solution to discourage the aggregation of the lipidated proteins. Following this step, the unbound protein was removed from the sample by passing the vesicles through Sephacryl S-1000 gel filtration media. The protein content of the resulting samples was then determined using SDS−PAGE. As shown in Figure 3b, none of the free protein samples were able to pass through the resin in the absence of SUVs, and vesicles exposed to unmodified **4a** carried no protein with them through the media. In contrast, SUVs were able to carry the lipid-modified proteins through the gel filtration column, suggesting that they had been embedded in the membrane bilayers. More detailed studies examining the nature of this association are in progress.

Through these studies, a new tyrosine modification reaction has been developed, capitalizing on the ability of transition metals to

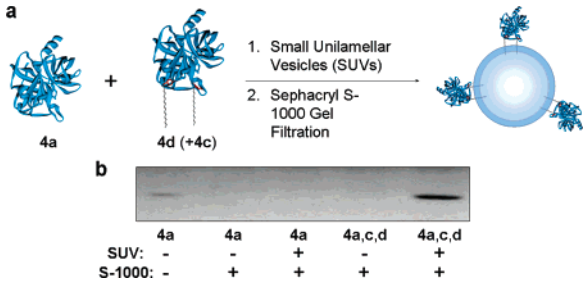

Figure 3. Incorporation of synthetic lipoproteins into lipid bilayers. A mixture of **4a**, **4c** (singly modified), and **4d** (doubly modified) was incubated with 100 nm small unilamellar vesicles (SUVs) for 20 min in the presence of 4 mM β-octyl-D-glucopyranoside. The unbound protein was removed by passing the vesicles through Sephacryl S-1000 gel filtration resin. (b) SDS−PAGE analysis indicated that only lipid-modified proteins were associated with the vesicles.

effect highly selective transformations through the activation of otherwise inert substrates. In addition to targeting an under-utilized functional group, this reaction provides an important step forward for the preparation of artificial lipoproteins. Current efforts are focusing on the use of alternative phosphine ligands to direct modifications to one of several tyrosine residues present on more complex protein targets.

Acknowledgment. We gratefully acknowledge the DOE Nanoscale Science, Engineering, and Technology Program for generous financial support, as well as the NIH (GM072700-01) and the University of California, Berkeley, Chemistry Department. We would also like to thank Jodi Gureasko and John Kuriyan for samples of H-Ras, and John Antos, Bill Galush, Bryan Jackson, and Jay Groves for helpful discussions.

Supporting Information Available: Full experimental procedures and characterization data for all intermediates. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA057106K