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Oxidative Modification of Native Protein Residues Using Cerium(IV) Ammonium Nitrate

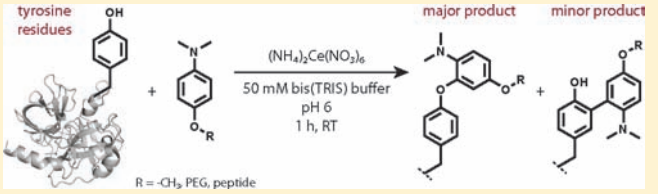
Kristen L. Seim,[†] Allie C. Obermeyer,[†] and Matthew B. Francis*[,†,‡]

[†]Department of Chemistry, University of California, Berkeley, California 94720-1460, United States

[‡]Materials Sciences Division, Lawrence Berkeley National Laboratories, Berkeley, California 94720-1460, United States

Supporting Information

ABSTRACT: A new protein modification strategy has been developed that is based on an oxidative coupling reaction that targets electron-rich amino acids. This strategy relies on cerium(IV) ammonium nitrate (CAN) as an oxidation reagent and results in the coupling of tyrosine and tryptophan residues to phenylene diamine and anisidine derivatives. The methodology was first identified and characterized on peptides and small molecules, and was subsequently adapted for protein modification by determining appropriate buffer conditions. Using the optimized procedure, native and introduced solvent-accessible residues on proteins were selectively modified with polyethylene glycol (PEG) and small peptides. This unprecedented bioconjugation strategy targets these under-utilized amino acids with excellent chemoselectivity and affords good-to-high yields using low concentrations of the oxidant and coupling partners, short reaction times, and mild conditions.



INTRODUCTION

The development of reliable, chemoselective reactions for protein modification is an important pursuit in the field of chemical biology, and these methods provide much-needed tools for the creation of protein-based materials.[1] Through the attachment of synthetic molecules to specific locations on protein surfaces, biomolecular structure and function can be both elucidated and exploited in a diverse number of contexts. To be useful in most cases, bioconjugation reactions must create precise, well-defined linkages without the use of protecting groups. In addition to the high degree of chemoselectivity, these reactions must proceed in aqueous solution under mild pH and temperature conditions and at low substrate concentrations. Although traditional bioconjugation strategies that target abundant amino acid side chains (such as lysine and aspartic and glutamic acids) are useful for nonspecific labeling, it is often difficult to control the number and locations of the modifications introduced at these sites.[1] When site specificity is required, the most utilized and reliable bioconjugation strategies target cysteine residues. These are particularly effective due to the low abundance[2] and nucleophilic nature of reduced thiolates on protein surfaces. However, there are many cases in which the introduction of a unique cysteine is inconvenient or difficult without disrupting protein function. Additionally, a growing number of protein-based materials require modification in two distinct locations, establishing a need for methods that can be combined with cysteine-targeting strategies to introduce multiple synthetic groups.[3] As one option, recent work has addressed these needs by introducing artificial amino acids and modifying the introduced functionalities with new protein-compatible chemistry.[4] While these approaches are very promising, there are still advantages to targeting native amino acids that can be introduced without specialized expression systems.

As an alternative solution to this problem, our group has explored the development of site-selective bioconjugation reactions that target the aromatic electron-rich amino acids tyrosine and tryptophan.[5−8] These amino acids occur with intermediate to low frequency on native protein surfaces[2] and are often partially or completely buried due to the amphiphilic nature of the phenolic and indole groups. Considering the complexities of these environments, it is therefore likely that individual, solvent-accessible tyrosine and tryptophan residues could be targeted though careful reagent design and selective optimization of reaction conditions. These amino acids, like cysteine, could also provide sites for selective modification when introduced on protein surfaces using site-directed mutagenesis. Despite their potential utility, broadly applicable, highly specific chemical reactions that target tyrosine and tryptophan have traditionally been lacking. Recent work has expanded the number of techniques for the modification of these amino acids, including the utilization of rhodium carbenoids[5,9,10] and malondialdehyde derivatives[11−13] for the alkylation of tryptophan and the use of Mannich-type additions,[7] π-allylpalladium complexes,[8] acyclic diazodicarboxamide reagents,[14] and diazonium salts[6] for the alkylation of tyrosine. Although the development of these techniques represents significant improvements in tryptophan- and tyrosine-targeting bioconjugation strategies, many of these methods would benefit from improved yields and lower levels of cross-reactivity.

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In order to develop new bioconjugation strategies that target these amino acids, our current studies have focused on taking advantage of the chemical properties of the electron-rich aromatic rings and their susceptibility to oxidation. Tyrosine and tryptophan residues have been shown to be preferred targets of reactive oxygen species, and several studies have documented the structures and consequences of oxidized residues in proteins.[15−17] Additionally, oxidized radical species of amino acids in biology are not only common, but are also crucial for many significant energy conversion processes.[18−21] The one-electron oxidation chemistry of tyrosine has been previously exploited by Kodadek and co-workers to cross-link proteins using metal-catalyzed oxidations.[22−24] Although the majority of these coupling reactions are hypothesized to occur through the addition of tyrosyl radicals to adjacent tyrosine residues, oxidized tryptophan radicals are also thought to play a role in some cases. One might expect that similarly generated amino acid radicals could add to electron-rich synthetic components, or conversely, the closed-shell forms of the amino acids could couple to free radicals in solution. Although the oxidation of other amino acids is well-documented and could pose a potential selectivity problem, we anticipated that reagents and conditions could be optimized for controlled and selective modifications that are limited to tyrosine and tryptophan residues.

In this article we describe an unprecedented oxidative coupling strategy that can selectively modify tyrosine and tryptophan residues using cerium(IV) ammonium nitrate (CAN) as a one-electron oxidant. This oxidative method was found to couple electron-rich aniline derivatives directly to tyrosine and tryptophan residues. This method was adapted for the chemoselective modification of proteins and was optimized to proceed with high yields at neutral pH and low substrate concentrations. The strategy has been used to modify both native and introduced residues on proteins with polyethylene glycol (PEG) and small peptides. The reaction was also used in conjunction with cysteine alkylation to doubly modify viral capsid proteins with both targeting and imaging functionalities.

RESULTS AND DISCUSSION

To determine compatible combinations of reactants and oxidants, a variety of candidates was screened for the modification of peptides in aqueous solution. To identify any reaction products that had formed, the reaction mixtures were analyzed using MALDI-TOF MS. The peptides used for screening were melittin as a tryptophan-containing peptide and angiotensin as a tyrosine-containing peptide, Figure 1a. These were combined with a series of electron-rich aromatic compounds and oxidants, such as ammonium persulfate, *N*-bromosuccinimide, *N*-chlorosuccinimide, Oxone, sodium periodate, potassium ferricyanide, and cerium(IV) ammonium nitrate (CAN). Initial screens identified CAN as a promising oxidant for the modification of these peptides, as this oxidant was found to efficiently couple aniline derivatives to one or both peptides. Anilines bearing additional electron-donating substituents (such as methoxy- and acetamido- groups) led to the highest levels of reactivity, while nitro- and halogen-substituted anilines were unreactive. Primary anilines generally lead to over-modification and complex product mixtures, likely due to the self-condensation reactions that occur with these species under oxidizing conditions. In contrast, *N*,*N*-dialkyl anilines bearing additional electron-donating substituents resulted in single additions to the peptides. One of the most promising combinations

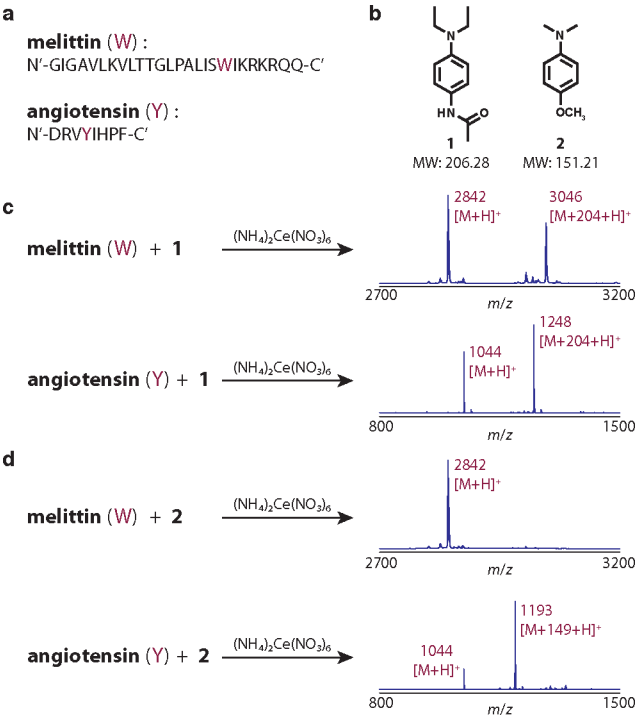

Figure 1. Modification of aromatic residues (W and Y) on (a) peptides using (b) phenylene diamine (**1**) and anisidine (**2**) derivatives in the presence of cerium(IV) ammonium nitrate (CAN). Reaction conditions: 1.5 mM CAN, 500 μM **1** or **2**, 100 μM peptide, 10 mM HEPES buffer, pH 4.5, 1 h. The reaction mixtures in (c) and (d) were analyzed using MALDI-TOF MS.

identified involved *N*-acyl phenylene diamine derivative 1, which was found to modify melittin and angiotensin a single time in the presence of CAN, Figure 1c.[25] MS/MS analysis of the modified peptides confirmed the chemoselectivity of these conditions for the modification of the tryptophan and tyrosine residues of melittin and angiotensin, respectively (Figures S1 and S2 in the Supporting Information [SI]). Alkylated anisidine **2** was also found to modify angiotensin in the presence of CAN, but this combination did not lead to product formation for melittin, Figure 1d.[26] This result was interpreted to indicate that the anisidine addition was selective for tyrosine residues. MS/MS analysis of the modified angiotensin was used to confirm this chemoselectivity (Figure S3 in the SI). In all cases, the mass changes observed suggested an addition of the small molecules to the indole and phenol rings with the loss of two hydrogen atoms. As a result of the promising levels of modification and clean reactivity observed with these reagents, the phenylene diamine and anisidine derivatives were carried on for further study.

To be broadly applicable for protein modification, bioconjugation reactions must proceed using buffered conditions that are close to neutral pH. However, the simple addition of CAN to unbuffered aqueous solution leads to significant acidification,[27] with a 1.5 mM solution of CAN in unbuffered H_2O reaching pH ~2−3. To counteract this unfavorable pH change, a series of buffers was screened, with the goal of maintaining neutral pH without precipitating or deactivating the Ce(IV) reagent.[28] These screens were performed in triplicate on melittin and angiotensin, and the relative product yields were determined using MALDI-TOF MS (see Figure S5 in the SI for response curves for quantitation by MALDI-TOF MS). The results are tabulated

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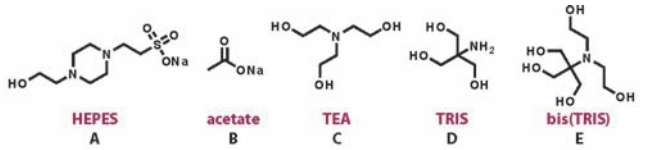

entry	buffer	buffer conc.	initial pH	final pH	1: % product melittin	1: % product angiotensin	2: % product melittin	2: % product angiotensin
1	A	10 mM	7	~4.5	35	50	0	80
2	A	50 mM	7	~6.5	0	5	0	5
3	A	100 mM	7	7	0	0	0	5
4	B	10 mM	5.5	5	25	50	0	55
5	B	50 mM	5.5	5.5	15	15	0	5
6	B	100 mM	5.5	5.5	10	5	0	5
7	C	10 mM	7	~4	20	90	0	95
8	C	50 mM	7	~6	0	10	0	20
9	C	100 mM	7	7	0	5	0	30
10	D	10 mM	7	~4	5	95	0	95
11	D	50 mM	7	~5	40	40	0	60
12	D	100 mM	7	6	0	20	0	35
13	E	10 mM	6	~5	30	75	0	45
14	E	50 mM	6	6	50	60	0	80
15	E	100 mM	6	6	15	45	0	60
16	E	10 mM	7	~6	0	25	0	30
17	E	50 mM	7	7	0	15	0	10
18	E	100 mM	7	7	0	5	0	10

Figure 2. Effects of buffer composition on pH stability and product formation. Reaction conditions: 1.5 mM CAN, 500 μM **1** or **2**, 100 μM peptide, RT, 1 h. Each reaction was repeated in triplicate and product ratios were determined using MALDI-TOF MS. Average product ratios are reported.

in Figure 2. Although a number of buffers were found to be compatible with the reaction, reactivity was typically lost as the strength of the buffers was increased to maintain mild pH conditions. For example, angiotensin could be modified in near-quantitative yield in 10 mM TRIS buffer (entry 10). However, the pH dropped to ~4 when the CAN was added at 1.5 mM (for representative MALDI-TOF MS spectra, see Figure S6 in the SI). Although most peptides can withstand these acidic conditions, proteins are more likely to denature at pH 4. For protein substrates, 50 mM bis(TRIS) was likely to be the optimal buffer, as it was able to maintain a pH of 6.0 with a minimal loss in reactivity (entry 14). In this buffer, a 100 μM solution of melittin was modified with a 50% conversion in 1 h using 1.5 mM CAN and 500 μM 1. Similarly, a 100 μM solution of angiotensin was modified with 60−80% conversion using 1.5 mM CAN and 500 μM 1 or 2 and the same reaction time (for representative MALDI-TOF MS spectra, see Figure S7 in the SI).

In order to characterize the reactivity observed on peptides, the structures of the modified amino acids were determined. To identify these modification products, we designed water-soluble, small-molecule tryptophan and tyrosine analogs that could be exposed to the reactive conditions used on peptides. The synthesized amino acid analogs (**3** and **5**) were subjected to the oxidative conditions in 50 mM bis(TRIS) buffer, pH 6.0. Any possible products were purified by flash chromatography and were analyzed by one-dimensional and two-dimensional NMR, as well as high-resolution mass spectrometry.

The coupling of **3** to **1** in the presence of CAN resulted in a single product (**4**) that could be isolated by flash chromatography. Two dimensional (2D) NMR analyses indicated that this compound resulted from an alkylation of the amide nitrogen of the phenylene diamine coupling partner, forming a carbon−nitrogen

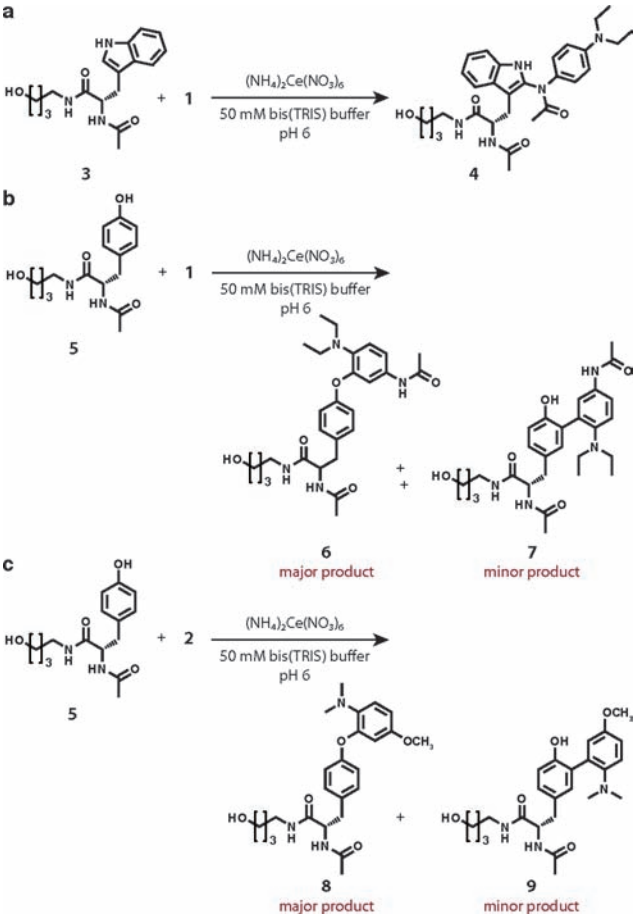

Figure 3. Reaction products obtained for small molecule (a) tryptophan and (b, c) tyrosine analogs. Product structures were determined using NOESY and HMBC NMR analyses (for spectra see SI).

bond on C-2 of the indole ring (Figure 3a; for 2D NMR spectra, see Figures S8 and S9 in the SI). High-resolution MS confirmed that the mass of the isolated small molecule corresponded to the structure that was identified using NMR, as well as to the mass change seen for melittin under analogous reaction conditions.

The reaction of **5** with **1** in the presence of CAN resulted in a 60:40 ratio of two isomeric products that could be separated by flash chromatography. High-resolution MS confirmed that the mass of both products corresponded to the mass changes observed for angiotensin under similar reactive conditions. Two-dimensional NMR analyses were used to identify the structural differences between these two products. The major product (**6**) was found to contain a bond between the tyrosine oxygen and an aromatic carbon on the phenylene diamine coupling partner. This bond was found to be ortho to the alkylated nitrogen on the phenylene diamine ring despite the steric demands of this substitution pattern. The minor product (**7**) was determined to be the result of carbon−carbon bond formation ortho to the phenolic oxygen of the tyrosine. This bond was also found to be located ortho to the alkylated nitrogen on the aromatic ring of the phenylene diamine coupling partner (Figure 3b; for 2D NMR spectra, see Figures S10−S13 in the SI).

When reacted with **2** in the presence of CAN, tyrosine analog **5** formed a 85:15 ratio of two isomeric products that could be separated by flash chromotography. These products were found to be structurally analogous to those identified in the

phenylene diamine reaction. The major product resulted from the formation of a similar carbon−oxygen bond, while the minor product resulted from the formation of an aromatic carbon−carbon bond (Figure 3c; for 2D NMR spectra see Figures S14−17 in the SI). As was the case for phenylene diamine **1**, each bond was formed at the position ortho to the alkylated nitrogen of the anisidine derivative. Additionally, the mass of the products corresponded to the identified structures and to the mass changes observed for angiotensin. Although the mechanisms for these modifications are currently under investigation, the formation of the observed products can be rationalized through radical coupling pathways (see Figure S18 in the SI for our current mechanistic hypotheses).

In order to evaluate the identified reactions on protein substrates, new tyrosine and tryptophan residues were introduced to the external surface of genome-free MS2 capsids. Composed of 180 monomers, MS2 is a porous, hollow structure with icosahedral symmetry.[29] After expression in *Escherichia coli,* the coat protein monomers spontaneously self-assemble into noninfectious capsids with 32 pores that allow access to the interior cavity. Although the sequence of wild-type MS2 includes a number of native tyrosine and tryptophan residues, the majority of these residues are not solvent-accessible and unlikely to modify with our coupling conditions. An exception is tyrosine 85, a residue on the interior surface of MS2 that has been successfully modified using alternative tyrosine modification strategies.[7] Although the presence of this internal tyrosine could result in double modification, we expected that an introduced tyrosine on the exterior surface could be selectively modified using a large molecular weight coupling partner (such as a modified PEG chain) that could not diffuse through the ∼2 nm pores. Additionally, if there were any difference in the solvent accessibilities of the two tyrosine residues, we might expect that the reaction conditions could be altered to achieve the selective modification of only the most solvent-accessible site.

Using site-selective mutagenesis, tyrosine and tryptophan residues were introduced into the external positions 19 and 15 of the MS2 monomer (Figure 4a). A non-natural *p*-aminophenol introduced at position 19 has been previously modified with high yields and selectivity with an alternative oxidative modification strategy.[4f] We therefore expected an introduced tyrosine or tryptophan at that position to be solvent-accessible and to be modified with similar success using the new Ce(IV)-based reaction. In addition, we chose to add the new amino acids at position 15, which has previously been shown to tolerate the introduction of a cysteine residue for use in subsequent alkylation reactions.[30] Located on the turn of a loop that extended from the external surface (Figure 4a), a tryptophan or tyrosine residue located at position 15 was likely to be solvent-accessible and might be expected to modify more readily than a tyrosine or tryptophan residue at position 19. Using the previously reported protocol for the purification of recombinantly expressed MS2 capsids,[4f] 60−90 mg of assembled protein could be obtained for each liter of cultured media. Although modestly lower than those obtained for wild-type MS2 (∼100 mg/L), these yields were significantly higher than those obtained when a non-natural residue is introduced to the same position (10−20 mg/L).

In order to apply our methodology toward the synthesis of useful bioconjugates and to evaluate the site-selectivity and biocompatibility of our modification strategy, we synthesized a series of modified PEGs (Figure 4b). In the presence of CAN, the PEG-substituted phenylene diamines led to the single modification of

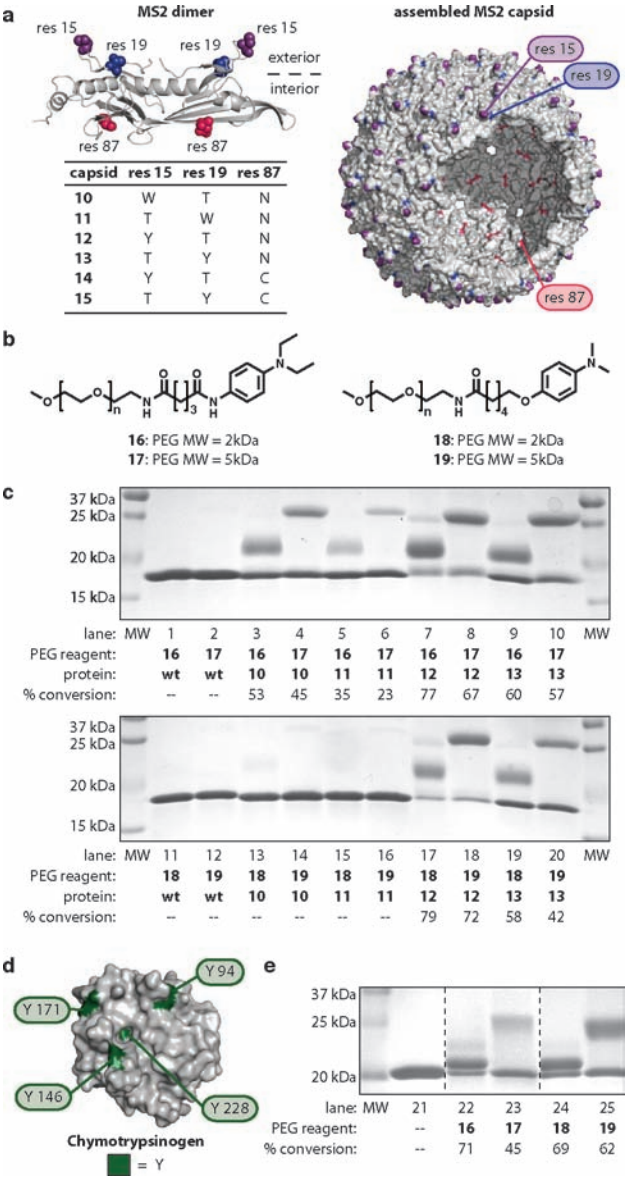

Figure 4. Oxidative coupling of PEG polymers to aromatic residues on protein substrates. (a) Six MS2 viral capsids were generated with tryptophan and tyrosine residues on the exterior surface of each monomer. Two of these capsids were also expressed with a solvent-accessible cysteine on the interior surface. (b) PEG-substituted phenylene diamine (**16**, **17**) and anisidine (**18**, **19**) derivatives were synthesized and exposed to each viral capsid in the presence of CAN. Reaction conditions: 1.5 mM CAN, 500 μM PEG, 25 μM protein, 50 mM bis(TRIS) buffer, pH 6, RT, 1 h. (c) Following capsid disassembly, the extent of modification for each reaction was determined using SDS-PAGE with Coomassie staining. (d) Native tyrosine residues (shown in green) were targeted on chymotrypsinogen under analogous conditions. (e) The extent of modification for chymotrypsinogen was determined using SDS-PAGE.

T15W (**10**), T19W (**11**), T15Y (**12**), and T19Y (**13**) MS2 capsids with high yields and short reaction times (Figure 4c). Wild-type MS2 was not modified under identical conditions, confirming that these modifications were dependent on the presence of the solvent-accessible, exterior tyrosine or tryptophan residues. Similarly, treatment of the MS2 mutants with the PEG-substituted anisidines resulted only in appreciable modification

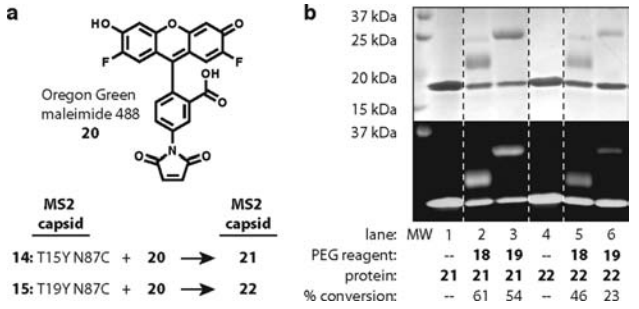

Figure 5. Double modification of MS2 viral capsids. (a) The cysteine residues located on the interior surface of capsids **14** and **15** were modified with Oregon Green maleimide 488 (**20**). (b) Fluorescently labeled proteins **21** and **22** were exposed to PEG-substituted anisidine derivatives (**18**, **19**) under reaction conditions analogous to those shown in Figure 4. Following capsid disassembly, the extent of modification for each reaction was determined using SDS-PAGE and visualization with fluorescent imaging and Coomassie staining.

of T15Y (**12**) and T19Y (**13**) MS2. Little to no modification of wild-type, T15W (**10**) or T19W (**11**) MS2 was observed. As was the case above, the selective modification of the tyrosine mutants with 18 and 19 occurred with high yields, short reaction times, and low concentrations of PEG coupling partners (500 μM). Additionally, analysis by size exclusion chromatography indicated that the capsids remained fully assembled during the modification process. Up to 77% conversion was observed (on the basis of densitometry analysis of the Coomassie-stained SDS PAGE gel), corresponding to the attachment of 138 polymer chains to each capsid.

Both the PEG-substituted phenylene diamine and anisidine reagents were also used to modify chymotrypsinogen, a protein with native, solvent-accessible tyrosine residues (Figure 4d). Analogous conditions resulted in high yields of modifications with all PEG substrates (Figure 4e), demonstrating the applicability of our modification strategy to a different protein substrate. In order to confirm the tyrosine selectivity of the reaction, chymotrypsinogen was reacted with small-molecule reagents 1 and 2. The resulting protein was subjected to trypsin digestion and analysis by LC−MS/MS. Only peptides fragments containing tyrosines were found to contain modifications (Figures S22 and S23 in the SI).

To explore the utility of our reaction for the double modification of proteins and, in particular, to investigate the compatibility of our reaction with cysteine alkylation, MS2 double mutants T15Y N87C (**14**) and T19Y N87C (**15**) were generated (Figure 4a). An introduced cysteine at position 87 has been found to be very reactive in previous work, and the internal surface of N87C MS2 capsids has been successfully modified with 100−180 copies of several different maleimide substrates.[4d,31−34] Although the oxidation of unprotected solvent-accessible cysteines and methionines has been observed in the presence of CAN (resulting in +16 amu adducts), we expected that the alkylation of the cysteines before exposure to the oxidative conditions could allow for the dual modification of the capsid. This would avoid oxidative deactivation of these introduced cysteine residues toward further modification with alkylation reagents. New cysteine residues were therefore introduced on the interior surface of capsids **12** and **13** at position 87, and the double mutants (**14** and **15**, respectively) were isolated with similar yields. To label the proteins with fluorescent dyes, the introduced cysteines in these capsids were

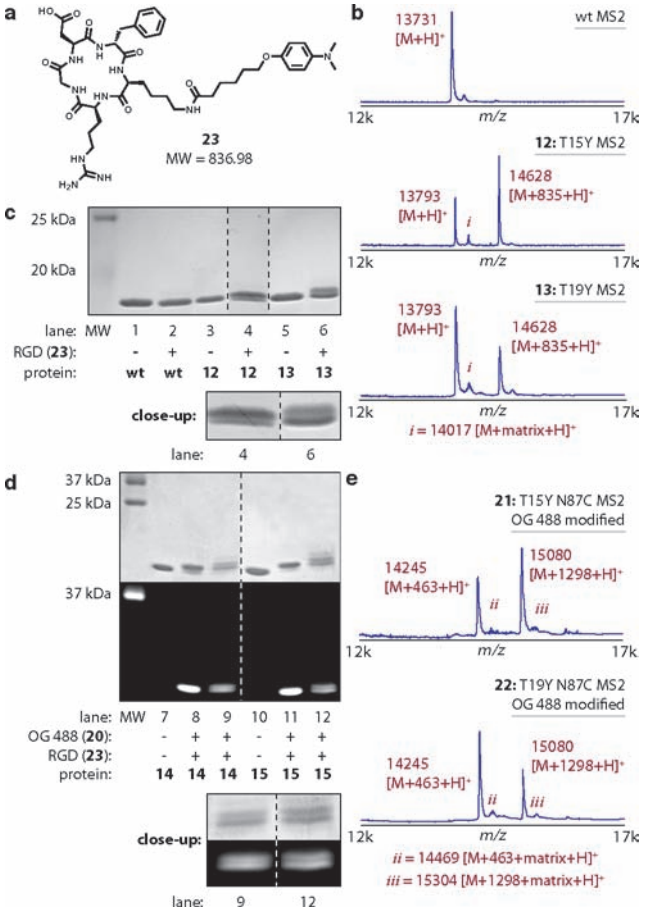

Figure 6. Oxidative coupling of a cell-targeting RGD peptide to tyrosine residues on the exterior surface of MS2 viral capsids. (a) An RGD-substituted anisidine derivative (**23**) was synthesized and exposed to each viral capsid in the presence of CAN. Reaction conditions: 1.25 mM CAN, 125 μM **23**, 25 μM protein, 50 mM bis(TRIS) buffer, pH 6, 1 h. (b, c) Following capsid disassembly, the extent of modification for each reaction was determined using SDS-PAGE and MALDI-TOF MS. (d, e) Fluorescently labeled MS2 was found to undergo modification with the RGD derivative under similar conditions. The extent of modification for each reaction was determined using SDS-PAGE and visualization with fluorescent imaging and Coomassie staining.

modified using Oregon Green 488 maleimide (**20**, Figure 5a). MALDI-TOF MS of the modified proteins (**21** and **22**) showed near-quantitative conversion to a single product (Figure S21 in the SI).

The treatment of fluorescently labeled proteins **21** (T15Y N87C) and **22** (T19Y N87C) with the PEG-substituted anisidine derivatives in the presence of CAN resulted in levels of modification that were comparable to the those observed previously. In these cases, the cysteine modifications appeared to be unperturbed by the oxidative conditions, as the PEG-modified proteins retained their fluorescence (Figure 5b). Reversing the reaction order for the dual modification of these proteins was also found to result in the differential modification of the tyrosine and cysteine groups, but lower yields of both reactions were observed. Presumably this was a result of the consumption of CAN by the oxidation of cysteines during the oxidative coupling step, and the resistance of any oxidized cysteines to modification with alkylation reagents. Therefore, as expected, in order to use this chemistry in combination with cysteine modification, it is best

to alkylate the thiol group before effecting the oxidative coupling method.

To install potential cancer targeting groups on the surface of MS2 capsids, small cell-targeting peptides were coupled to the introduced tyrosine residues using the oxidative coupling strategy. Cyclic RGD is a widely used peptide for the binding of integrin $\alpha_v\beta_3$.[35−37] A five-amino acid RGD-substituted anisidine (**23**, Figure 6a) was synthesized and was shown to couple to T15Y (**12**) and T19Y (**13**) MS2 with high yields and selectivity (Figure 6b,c). Little to no modification was observed for wild-type MS2, indicating that the solvent-accessible, exterior tyrosine was needed for the reaction to occur. Exposure of fluorescently labeled capsids **21** and **22** to the RGD-substituted anisidine using analogous reaction conditions also resulted in modification, generating assemblies that were functionalized with both an internal imaging group and an exterior targeting group (Figure 6d,e). Although the application of this methodology for the attachment of peptides to proteins will be limited to sequences without tyrosine residues, the ability of this unprecedented reaction to reach appreciable levels of conversion despite the significant amount of steric hindrance suggests that it will find use in many circumstances.

CONCLUSION

This work introduces several new oxidative coupling methods for the chemoselective modification of aromatic amino acids in proteins. These reactions have not been reported previously, and thus add to the important and growing list of useful bioconjugation techniques. The ability of these reactions to target natural amino acids provides a useful complement to strategies that require the use of artificial amino acids. Similar levels of chemoselectivity and yield can be achieved, provided that there are few surface-accessible tyrosines and tryptophans in the protein substrate. Although not the focus of this work, the new tryptophan reactivity provides very important design leads for future reactions, as there are few existing bioconjugation methods for this residue.[5,9−13] In comparison to other tyrosine modification strategies, the anisidine coupling reaction proceeds with excellent chemoselectivity and moderate to low concentrations of simple reagents. It avoids the elevated pH required for diazonium coupling reactions and does not modify histidine residues.[1] Futhermore, it generally reaches higher levels of conversion in shorter time periods than previously reported Mannich-type couplings[7] and π-allylpalladium alkylations.[8] We therefore envision these new oxidative coupling reactions to be useful in many situations that require bioconjugation reactions to be run on large scale, such as the creation of new protein-based materials.

ASSOCIATED CONTENT

Supporting Information. Full experimental details, additional characterization spectra, protein digest information, and current mechanistic hypotheses for these transformations. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author
francis@cchem.berkeley.edu

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REFERENCES

(1) (a) Hermanson, G. T. *Bioconjugate Techniques*, 2nd ed.; Academic Press: San Diego, 2008. (b) Tilley, S. D.; Joshi, N. S.; Francis, M. B. The Chemistry And Chemical Reactivity Of Proteins. In *The Wiley Encyclopedia of Chemical Biology*; Begley, T., Ed; Wiley-VCH: Weinheim, 2008.

(2) Doolittle, R.F. R. Redundancies in Protein Sequence. In *Prediction of Protein Structures and the Principles of Protein Conformation*; Fasman, G. D., Ed; Plenum Press: New York, 1989.

(3) Examples of applications that require modifications in two or more distinct locations include Förster resonance energy transfer (FRET) experiments,[3a] folding studies,[3b] or the dual modification of proteins for the targeted delivery of drugs or imaging agents.[3c,d](a) Joo, C.; Balci, H.; Ishitsuka, Y.; Buranachai, C.; Ha, T. *Annu. Rev. Biochem.* **2008**, *77*, 51–76. (b) Cecconi, C.; Shank, E. A.; Bustamante, C.; Marqusee, S. *Science* **2005**, *309*, 2057–2060. (c) Banerjee, D.; Liu, A. P.; Voss, N. R.; Schmid, S. L.; Finn, M. G. *ChemBioChem* **2010**, *11*, 1273–1279. (d) Stephanopoulos, N.; Tong, G. J.; Hsiao, S. C.; Francis, M. B. *ACS Nano* **2010**, *4*, 6014–6020.

(4) Examples of adding new functionality to artificial amino acids have involved methods targeting ketones,[4a] azides,[4b,c,d] alkynes,[4b,d,e] and anilines.[4f,g](a) Cornish, V. W.; Hahn, K. M.; Schultz, P. G. *J. Am. Chem. Soc.* **1996**, *118*, 8150–8151. (b) Deiters, A.; Cropp, T. A.; Mukherji, M.; Chin, J. W.; Anderson, J. C.; Schultz, P. G. *J. Am. Chem. Soc.* **2003**, *125*, 11782–11783. (c) Kiick, K. L.; Saxon, E.; Tirrell, D. A.; Bertozzi, C. R. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 19–24. (d) Strable, E.; Prasuhn, D. E.; Udit, A. K.; Brown, S.; Link, A. J.; Ngo, J. T.; Lander, G.; Quispe, J.; Potter, C. S.; Carragher, B.; Tirrell, D. A.; Finn, M. G. *Bioconjugate Chem.* **2008**, *19*, 866–875. (e) van Hest, J. C. M.; Kiick, K. L.; Tirrell, D. A. *J. Am. Chem. Soc.* **2000**, *122*, 1282–1288. (f) Carrico, Z. M.; Romanini, D. W.; Mehl, R. A.; Francis, M. B. *Chem. Commun.* **2008**, 1205–1207. (g) Mehl, R. A.; Anderson, J. C.; Santoro, S. W.; Wang, L.; Martin, A. B.; King, D. S.; Horn, D. M.; Schultz, P. G. *J. Am. Chem. Soc.* **2003**, *125*, 935–939.

(5) Antos, J. M.; Francis, M. B. *J. Am. Chem. Soc.* **2004**, *126*, 10256–10257.

(6) Hooker, J. M.; Kovacs, E. W.; Francis, M. B. *J. Am. Chem. Soc.* **2004**, *126*, 3718–3719.

(7) Joshi, N. S.; Whitaker, L. R.; Francis, M. B. *J. Am. Chem. Soc.* **2004**, *126*, 15942–15943.

(8) Tilley, S. D.; Francis, M. B. *J. Am. Chem. Soc.* **2006**, *128*, 1080–1081.

(9) Antos, J. M.; McFarland, J. M.; Iavarone, A. T.; Francis, M. B. *J. Am. Chem. Soc.* **2009**, *131*, 6301–6308.

(10) Popp, B. V.; Ball, Z. T. *J. Am. Chem. Soc.* **2010**, *132*, 6660–6662.

(11) Foettinger, A.; Leitner, A.; Lindner, W. *J. Proteome Res.* **2007**, *6*, 3827–3834.

(12) Foettinger, A.; Melmer, M.; Leitner, A.; Lindner, W. *Bioconjugate Chem.* **2007**, *18*, 1678–1683.

(13) Sturm, M.; Leitner, A.; Lindner, W. *Bioconjugate Chem.* **2011**, *22*, 211–217.

(14) Ban, H.; Gavrilyuk, J.; Barbas, C. F. *J. Am. Chem. Soc.* **2010**, *132*, 1523–1525.

(15) Davies, M. J. *Biochim. Biophys. Acta-Proteins Proteomics* **2005**, *1703*, 93–109.

(16) Davies, M. J. *Biochem. Biophys. Res. Commun.* **2003**, *305*, 761–770.

(17) Dean, R. T.; Fu, S.; Stocker, R.; Davies, M. J. *Biochem. J.* **1997**, *324*, 1–18.
(18) Ahlbrink, R.; Haumann, M.; Cherepanov, D.; Bögershausen, O.; Mulkidjanian, A.; Junge, W. *Biochemistry* **1998**, *37*, 1131–1142.
(19) Hoganson, C. W.; Babcock, G. T. *Science* **1997**, *277*, 1953–1956.
(20) Renger, G. *Biochim. Biophys. Acta-Bioenergetics* **2004**, *1655*, 195–204.
(21) Stubbe, J.; van Der Donk, W. A. *Chem. Rev.* **1998**, *98*, 705–762.
(22) Brown, K. C.; Kodadek, T. *Met. Ions Biol. Syst.* **2001**, *38*, 351–384.
(23) Fancy, D. A.; Denison, C.; Kim, K.; Xie, Y.; Holdeman, T.; Amini, F.; Kodadek, T. *Chem. Biol.* **2000**, *7*, 697–708.
(24) Fancy, D. A.; Kodadek, T. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 6020–6024.
(25) We have previously shown that *N*,*N*-dialkylphenylene diamines and anisidine derivatives couple to introduced aniline groups on proteins in the presence of sodium periodate.[2,5a,b] These conditions lead to minimal if any modification of native residues. See Figure S4 in the Supporting Information for MALDI-TOF MS spectra of melittin and angiotensin after subjection to analogous coupling conditions using sodium periodate as the oxidant. (a) Hooker, J. M.; Esser-Kahn, A. P.; Francis, M. B. *J. Am. Chem. Soc.* **2006**, *128*, 15558–15559. (b) Stephanopoulos, N.; Carrico, Z. M.; Francis, M. B. *Angew. Chem., Int. Ed.* **2009**, *48*, 9498–9502. See also reference 4f.
(26) Although the use of cerium (IV) ammonium nitrate has been reported for the deprotection of *p*-methoxy benzyl ethers in aqueous solutions,[26a,b] we found our anisidine derivatives to be stable in the presence of CAN over a period of 5 h. (a) Classon, B.; Garegg, P. J.; Samuelsson, B. *Acta Chem. Scand. B* **1984**, *B38*, 419–422. (b) Johansson, R.; Samuelsson, B. *J. Chem. Soc., Perkin Trans. 1* **1984**, 2371–2374.
(27) For a general reference on cerium chemistry, see: Binnemans, K. Applications of Tetravalent Cerium Compounds. In *Handbook on the Physics and Chemistry of Rare Earths*; Gschneidner, K. A., Jr., Bünzli, J.-C. G., Pecharsky, V. K., Eds; Elsevier B.V.: Amsterdam, 2006.
(28) Phosphate buffer was not screened; the addition of cerium(IV) ammonium nitrate to phosphate buffer solutions results in the immediate precipitation of the cerium species. As result, the oxidant is deactivated, and the coupling reactions are unable to proceed. For the successful application of this methodology, it is therefore necessary to remove any phosphate from the reaction mixture.
(29) Valegard, K.; Liljas, L.; Fridborg, K.; Unge, T. *Nature* **1990**, *345*, 36–41.
(30) Peabody, D. S. *J. Nanobiotechnol.* **2003**, *1*, 5.
(31) Meldrum, T.; Seim, K. L.; Bajaj, V. S.; Palaniappan, K. K.; Wu, W.; Francis, M. B.; Wemmer, D. E.; Pines, A. *J. Am. Chem. Soc.* **2010**, *132*, 5936–5937.
(32) Stephanopoulos, N.; Carrico, Z. M.; Francis, M. B. *Angew. Chem., Int. Ed.* **2009**, *48*, 9498–9502.
(33) Tong, G. J.; Hsiao, S. C.; Carrico, Z. M.; Francis, M. B. *J. Am. Chem. Soc.* **2009**, *131*, 11174–11178.
(34) Wu, W.; Hsiao, S. C.; Carrico, Z. M.; Francis, M. B. *Angew. Chem., Int. Ed.* **2009**, *48*, 9493–9497.
(35) Pierschbacher, M. D.; Ruoslahti, E. *Nature* **1984**, *309*, 30–33.
(36) Auzzas, L.; Zanardi, F.; Battistini, L.; Burreddu, P.; Carta, P.; Rassu, G.; Curti, C.; Casiraghi, G. *Curr. Med. Chem.* **2010**, *17*, 1255–1259.
(37) Liu, S. *Mol. Pharmaceutics* **2006**, *3*, 472–487.