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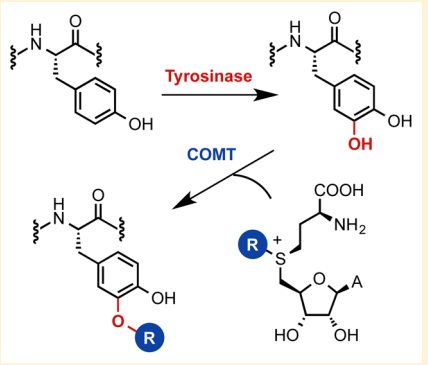
An Enzyme Cascade for Selective Modification of Tyrosine Residues in Structurally Diverse Peptides and Proteins

Anna-Winona Struck,[†] Matthew R. Bennett,[†] Sarah A. Shepherd, Brian J. C. Law, Ying Zhuo, Lu Shin Wong, and Jason Micklefield*

School of Chemistry and Manchester Institute of Biotechnology, The University of Manchester, 131 Princess Street, Manchester M1 7DN, United Kingdom

Supporting Information

ABSTRACT: Bioorthogonal chemistry enables a specific moiety in a complex biomolecule to be selectively modified in the presence of many reactive functional groups and other cellular entities. Such selectivity has become indispensable in biology, enabling biomolecules to be derivatized, conjugated, labeled, or immobilized for imaging, biochemical assays, or therapeutic applications. Methyltransferase enzymes (MTase) that accept analogues of the cofactor *S*-adenosyl methionine have been widely deployed for alkyl-diversification and bioorthogonal labeling. However, MTases typically possess tight substrate specificity. Here we introduce a more flexible methodology for selective derivatization of phenolic moieties in complex biomolecules. Our approach relies on the tandem enzymatic reaction of a fungal tyrosinase and the mammalian catechol-*O*-methyltransferase (COMT), which can effect the sequential hydroxylation of the phenolic group to give an intermediate catechol moiety that is subsequently *O*-alkylated. When used in this combination, the alkoxylation is highly selective for tyrosine residues in peptides and proteins, yet remarkably tolerant to changes in the peptide sequence. Tyrosinase−COMT are shown to provide highly versatile and regioselective modification of a diverse range of substrates including peptide antitumor agents, hormones, cyclic peptide antibiotics, and model proteins.



INTRODUCTION

The introduction of bioorthogonal functional groups into biomolecules has found widespread application in biomolecular conjugation for imaging, therapeutics, and pull-down assays.[1−5] For example, biomolecules that have been derivatized with terminal alkynyl groups can undergo highly efficient and selective Cu(I)-catalyzed cycloaddition reactions, with azide-functionalized coupling partners in a reaction that is entirely orthogonal to any other biological entities.[1−4] The exquisite selectivity of enzymes, coupled with the capability to reengineer their properties, offers a powerful means to introduce bioorthogonally reactive functionality into biomolecules of interest.[6−14] To this end, a number of enzymatic reactions have been exploited to selectively label biomolecules including peptides, proteins, nucleic acids, and glycans.[6−17]

One class of enzymes that has proven to be particularly versatile for functionalization of small metabolites through to biopolymers are the *S*-adenosyl methionine (AdoMet)-dependent methyltransferases (MTases).[15] While most MTases tend to exhibit tight substrate specificity, several members of this family exhibit more relaxed cofactor specificity and will accept AdoMet analogues with alternative *S*-alkyl sulfonium groups, transferring a range of different alkyl groups including bioorthogonal functional groups.[16−30] Consequently, MTases along with AdoMet analogues are now widely used to label DNA,[16−19] RNA,[20−22] and histone proteins.[23−25] AdoMet analogues have also been used to alkylate and label natural product scaffolds[26−29] including the antitumor agent rebeccamycin[28] and the potent immunosuppressive agent rapamycin.[29] Small molecule alkylations through this approach can thus be used to modulate the physicochemical and biological activity of clinically important natural products.

Although nature has evolved MTases for a large number of substrates, there are still many potential substrates where regioselective alkylation would be desirable, for which no cognate MTase exists. For example, a number of protein methyltransferases (PRMTs) have been characterized that can alkylate Lys and Arg residues within conserved peptide sequences of histones.[23−25] However, MTases that can methylate alternative nucleophilic amino acid residues in other proteins are rare. In light of this, we set out to develop an alternative and more generic MTase based bioalkylation system. This system would recognize and modify a specific and unique functional group within a range of proteinogenic and nonproteinogenic peptide scaffolds. To this end, we identified catechol-*O*-methyl-transferase (COMT), an MTase with more relaxed substrate specificity, as a potential bioalkylation tool. COMT accepts a range of catecholamines, including L-3,4-dihydroxyphenylalanine (L-DOPA),[30] a nonproteinogenic

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amino acid used in the treatment of Parkinson's disease.[31] X-ray crystal structures of COMT[32,33] show substituents that are attached to the substrate catechol moiety protrude from the active site out into the solvent (Figure S1); we therefore reasoned that COMT may be capable of accepting L-DOPA containing peptides and proteins. L-DOPA is known to replace tyrosine in proteins, particularly in response to oxidative stress or when administered for the treatment of Parkinson's disease.[34−39] However, the catechol moiety is intrinsically unstable and is easily oxidized to highly reactive *ortho*-quinone species, which react nonselectively with nucleophilic groups leading to unwanted aggregation and cross-linked adducts.[40−42] Therefore, we envisaged a strategy to convert tyrosine residues to L-DOPA by enzymatic hydroxylation, followed by rapid COMT-mediated alkylation (Figure 1). An ideal hydroxylase−

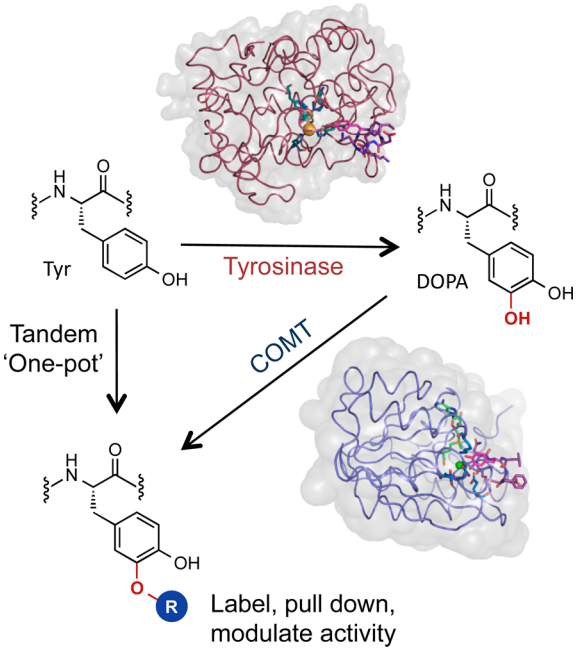

Figure 1. A general strategy for regioselective derivatization of tyrosine residues in peptides and proteins by a tandem enzymatic alkoxylation. Tyrosinase hydroxylates tyrosine to give an intermediate L-DOPA residue, which is then alkylated by catechol-*O*-methyltransferase (COMT) with AdoMet or AdoMet analogues.

COMT system should be specific for tyrosine over other amino acid residues. However, unlike the native protein MTases, which only recognize conserved peptide sequences, the coupled enzyme reaction would be tolerant to changes in the peptide sequence and structure. In this way, a greater diversity of tyrosine containing substrates might be selectively alkylated.

RESULTS AND DISCUSSION

COMT-Catalyzed *O*-Methylation of L-DOPA-Containing Peptides. It has been known for some time that L-DOPA is a substrate for COMT.[43] However, as far as we are aware, there are no reports of COMT-catalyzed methylation of L-DOPA residues that were incorporated into peptides or proteins. Accordingly, two model peptides containing an internal and a terminal L-DOPA (Y^{OH}) residue were synthesized (Figure 2). Peptide KNFLDY^{OH} (**1a**), containing a C-terminal L-DOPA residue, is derived from a peptide sequence present in peptidyl-prolyl isomerases (PpiB).

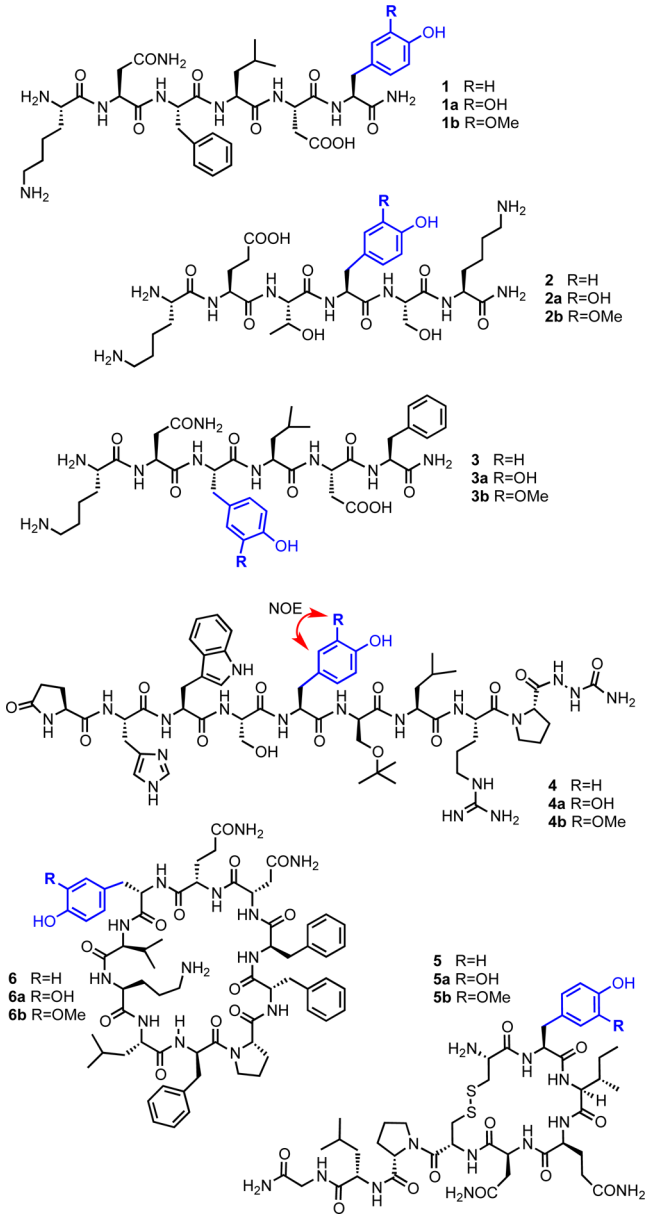

Figure 2. Structures of peptide substrates (**1**−**6**), hydroxylated products (**1a**−**6a**) of tyrosinase, and methoxylated products (**1b**−**6b**) from tandem tyrosinase−COMT reactions.

Previous studies have shown that L-DOPA is incorporated into PpiB instead of Tyr, if an *Escherichia coli* cell-free transcription/translation system is supplemented with L-DOPA.[36,37] The peptide KETY^{OH}SK (**2a**), with an internal L-DOPA residue, is derived from another naturally occurring L-DOPA peptide identified previously.[44] Methylation assays with both peptides, COMT and AdoMet as a methyl donor were performed. Analysis after 60 min by MALDI-TOF MS and HPLC showed that KNFLDY^{OH} (**1a**) was completely methylated to KNFLDY^{OMe} (**1b**), while conversion of KETY^{OH}SK (**2a**) to the methylated product KETY^{OMe}SK (**2b**) reached 55% over the same time period. This suggests that the sequence of the peptide has influence on the methylation reactions, with the more flexible and accessible terminal L-DOPA residue preferred by COMT.

The two methylated peptides (**1b** and **2b**) were subsequently isolated and characterized by MS−MS and TOCSY-NMR. The

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y and b fragment ions are entirely consistent with the inclusion of a methylated-DOPA residue at the corresponding position in the peptide sequences for (**1b**) (Figure S4). TOCSY experiments also allowed complete assignment of ^{1}H NMR spectra, including singlets at δ 3.73 ppm for methoxy group of both products (Figures S5 and S10). With smaller catecholamine substrates, COMT has been shown to give predominately *meta*-methylation products with a minor amount of the *para*-methylated regioisomers.[45−47] X-ray crystal structures of COMT[32,33,47] indicate that for larger catechol substrates the *meta*-methylation binding mode is preferred; in the case of L-DOPA peptides, the peptide backbone is most likely to be orientated out into the solvent which positions the catechol *meta*-hydroxyl adjacent to AdoMet (Figure S1).

Tyrosinase-Catalyzed Hydroxylation of Linear and Cyclic Peptide Substrates. The copper-dependent enzyme tyrosinase utilizes molecular oxygen to hydroxylate L-tyrosine to give L-DOPA and also catalyzes the subsequent oxidation of the catechol moiety resulting in an *ortho*-quinone species.[48,49] However, it is known that overoxidation to the undesired *ortho*-quinone can be suppressed with the addition of ascorbic acid.[44] To further explore the substrate specificity of tyrosinase, several peptide sequences were synthesized including KNFLDY (**1**), KETYSK (**2**) and KNYLDF (**3**). Hydroxylation assays with tyrosinase and ascorbic acid resulted in good conversions to the corresponding hydroxylated peptides (**1a**, **2a**, and **3a**, Figure 3). Product peptides (**1a** and **2a**) were identical to the synthetic standards by HPLC and MS, while MS analysis of (**3a**) was also consistent with hydroxylation of the tyrosine residue (Figure S14). These results indicate that tyrosinase can hydroxylate both internal and terminal amino acid residues. The peptide with a terminal tyrosine residue (**1**) was hydroxylated fastest affording 97% of **1a** after 15 min, while peptides with internal tyrosine residues (**2** and **3**) gave ca. 62−64% of hydroxylated products (**2a** and **3a**) over the same time period (Figure 3), presumably due to the steric constraints of accessing the internal residues.

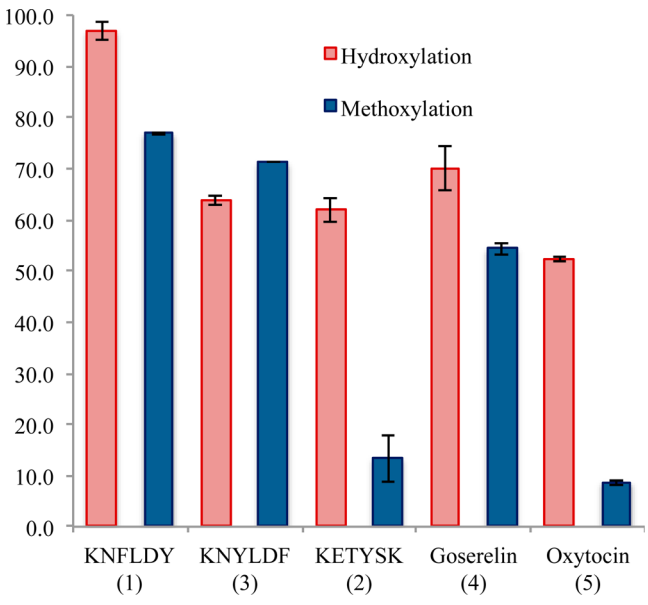

Figure 3. Percent conversion of peptides **1**−**5** to hydroxylated products **1a**−**5a** (red bars) and methoxylated products **1b**−**5b** (blue bars). For hydroxylation assays, peptide substrate (0.5 mM) was incubated at 30 °C for 15 min with mushroom tyrosinase (0.2 μM), $MgCl_2$ (3 mM), and ascorbic acid (12.5 mM) in a 20 mM potassium phosphate buffer at pH 7.4. Tandem methoxylation assays with peptides **1**−**5** were carried out under the same conditions except COMT (15 μM) and AdoMet (2 mM) were also included and reactions were incubated for an extended period of 5 h.

More challenging and biologically important peptides goserelin (**4**),[50] oxytocin (**5**),[51] and tyrocidine A (**6**)[52] were also tested as possible tyrosinase substrates. The synthetic decapeptide goserelin (Zoladex) is used in the treatment of prostate cancer. The human hormone oxytocin, a cyclic nonapeptide, is a WHO essential medicine and along with synthetic analogues has found widespread medical use.[53] Finally, the cyclic nonribosomal peptide antibiotic tyrocidine A was selected as one example of many tyrosine containing bioactive secondary metabolites.[54] All three peptides (**4**, **5**, and **6**) were monohydroxylated by tyrosinase, with MS−MS analysis of the products **4a**, **5a**, and **6a** indicating completely regioselective hydroxylation of the desired tyrosine residues (Figures S18, S28, S29, and S34). As anticipated, tyrosinase hydroxylation was faster for the linear peptide goserelin (**4**) than for the more conformationally constrained cyclic peptides **5** and **6**. When the incubation time of oxytocin (**5**) was extended from 15 min, under the standard conditions described in Figure 3, to 120 min, it was possible to increase the percent hydroxylation from 52% to near full conversion (94%). However, extended incubation periods did not lead to a significant increase for the hydroxylation of tyrocidine A (**6**). This is likely due to the fact that tyrocidine A has been shown to dimerize in solution.[55] A recent X-ray crystal structure of the tyrocidine A homodimer reveals that the side chain of the tyrosine is in close proximity to the side chains of the adjacent glutamine and a D-phenylalanine residue from the opposite subunit, which could decrease the flexibility and accessibility of the phenolic group.[56]

To further compare the selectivity of tyrosinase, a substrate KNYLDY (**7**) with both internal and terminal tyrosine residues was synthesized and tested. HPLC and MS experiments revealed that this peptide **7** was hydroxylated to give a single major product (**7a**) in ca. 63% yield after 3 min (Figure 4A), and MS−MS analysis revealed the major product possessed a terminal L-DOPA residue, with only a minor amount of internal hydroxylation observed (Figure S39). This data suggests that tyrosinase is selective for tyrosine residues that occupy more accessible and flexible positions at the termini of peptide substrates.

Tandem Enzymatic Hydroxylation and Alkylation Reactions. Having established tyrosinase and COMT activity with a range of peptide substrates, we next sought to determine if the two enzymes were compatible together *in vitro*. Accordingly, the peptides KNFLDY (**1**), KETYSK (**2**), KNYLDF (**3**), goserelin (**4**), oxytocin (**5**), and tyrocidine A (**6**) were each incubated with tyrosinase, ascorbic acid, COMT, and AdoMet. In all cases, monomethoxylated peptides (**1b**−**6b**) were produced. The methoxylated products were characterized by MS−MS and by ^{1}H NMR with assignments obtained by TOCSY showing characteristic methoxy singlets between δ 3.7 and 3.8 ppm for the new peptide products. Additional NOESY experiments with methoxygoserelin (**4b**) also confirmed that the methylation had occurred at the *meta*-hydroxyl group (Figure S22); a NOE was observed between the methoxy (δ 3.74 ppm) and the C2 aryl (δ 6.61 ppm) proton singlets. This result confirmed not only that the hydroxylation was regioselective, but also that the methylation was

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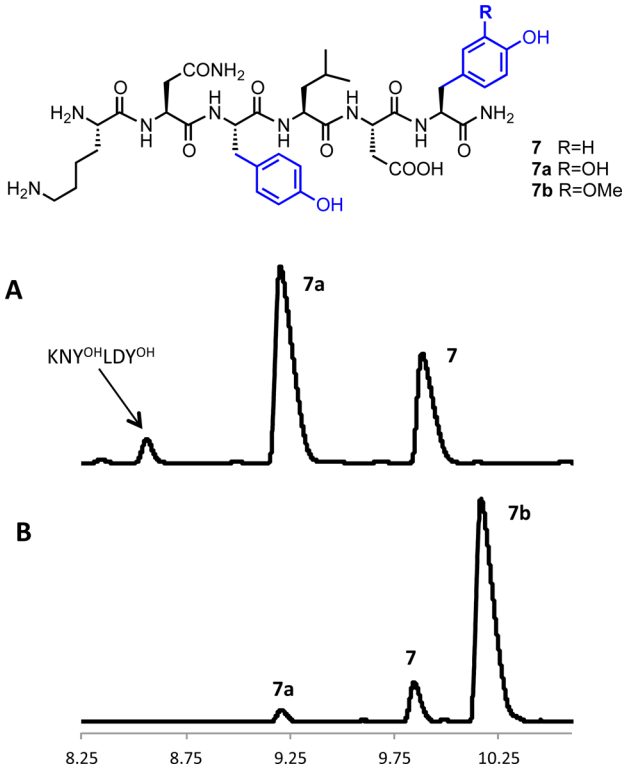

Figure 4. HPLC chromatograms from the hydroxylation and methoxylation of 7. (A) Hydroxylation of 7 was carried out using the standard procedure (see Figure 3) at 30 °C over a reduced time period of 3 min. (B) Methoxylation of 7 followed the standard conditions, except incubation with tyrosinase was reduced to 3 min, at which point tyrosinase was removed using a 50 kDa MWCO-filter before incubation with COMT (60 μM) and AdoMet (3 mM) for 4 h. For MS–MS analysis see Figures S39 and S41.

regioselective for the *meta*-hydroxyl group of hydroxygoserelin (**4a**), as anticipated.

To maximize the yields of the methoxylation products, the conditions of the tandem tyrosinase–COMT reaction were optimized. A temperature of 30 °C was used, which was a compromise between the optimum for each enzyme. DTT was excluded as this can interfere with the tyrosinase catalyzed reaction,[57] and the concentration of ascorbic acid was minimized as this is methylated by COMT.[58] It was also apparent that while the hydroxylation reaction was rapid, the subsequent methylation reaction required an extended time-period of 5 h to reach higher conversions. As anticipated, the highest conversions were obtained for the linear substrate with a terminal tyrosine residue (**1**), affording 76% of **1b** (Figure 3). Substrates with internal tyrosine residues (**2**–**4**) afforded methoxylated products with conversions between 13% and 71%, while cyclic peptides oxytocin (**5**) and tyrocidine (**6**) gave low yields of methoxylated products (**5b** and **6b**) under the standard conditions (Figure 3). Although the methoxylation of oxytocin (**5**) and tyrocidine (**6**) was slow, the cyclic products (**5b** and **6b**) were nevertheless isolated after extended incubation periods. By extending the incubation time of oxytocin (**5**) with tyrosinase and COMT from 5 to 16 h, we were able to increase the extent of methoxylation from 9% to 65%. This indicates that there is scope to further optimize the tandem enzymatic reaction for specific peptide substrates.

The methoxylation of KNYLDY (**7**), possessing both internal and terminal tyrosine residues, was also explored (Figure 4B). In this case, it was possible to obtain selective methoxylation of the terminal tyrosine (**7b**), by reducing the incubation period with tyrosinase to 3 min and then removing tyrosinase using a 50 kDa MWCO filter, prior to incubation with COMT and AdoMet. MS–MS analysis (Figures S40 and S41) confirmed the sequence of (**7b**), with only a minor amount of a product resulting from methoxylation of the internal tyrosine residue evident. The preference of the tandem enzymatic reaction for more accessible tyrosine residues could potentially enable selective labeling of substrates with more than one tyrosine. This preference, for example, may enable selective labeling of terminal tyrosines in proteins.

Installing Bioorthogonal Functionality into Peptides Using Tyrosinase and COMT. Having shown selective hydroxylation and methylation of tyrosine containing peptides, we next sought to establish if larger functionalized groups could be transferred from an AdoMet analogue to peptide substrates. Here we chose to test the *S*-4-propargyloxybut-2-enyl analogue of AdoMet (POB-AdoMet, Figure 5) which has been shown to function as a cofactor with PRMTs.[23,24] The terminal alkyne group enables labeling, pull down, and immobilization via cycloaddition reactions with azido functionalized coupling partners. Previously, COMT was shown to accept a propan-

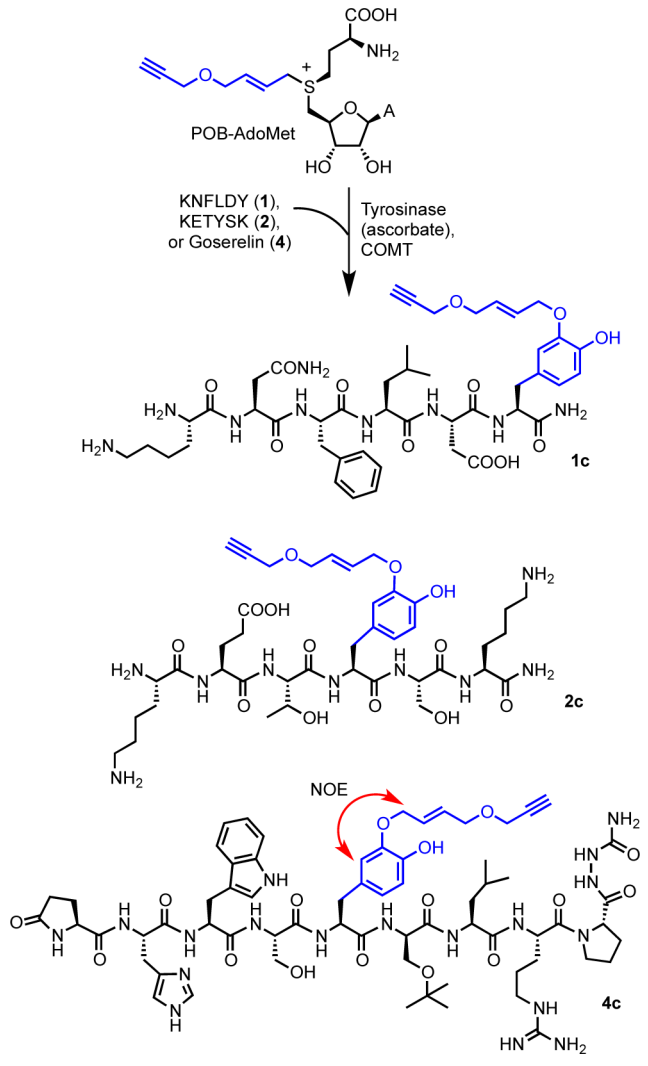

Figure 5. Introduction of bioorthoganal functionality into peptides using tyrosinase–COMT and POB-AdoMet.

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2-one analogue of AdoMet, transferring the ketone functional group to small catechol substrates.[59] Given that POB-AdoMet is larger than the propan-2-one[59] and most other analogues of AdoMet that have been used to date,[15] it was necessary to establish if COMT, in addition to exhibiting considerable substrate flexibility, can also tolerate significant changes to the AdoMet cofactor. Accordingly, KNFLDY (**1**) and KETYSK (**2**) were first hydroxylated with the tyrosinase and the L-DOPA peptides (**1a** and **2a**) were incubated with COMT and POB-AdoMet for 1 h. MALDI MS and MS−MS experiments (Figures S6 and S12) showed that the POB group is transferred to give POB-peptides (**1c** and **2c**) (Figure 5).

To demonstrate that the POB-chain can also be transferred to a more complex substrate, goserelin (**4**) was hydroxylated with tyrosinase and incubated with COMT and POB-AdoMet. After optimization of reaction conditions, the POB-goserelin derivative (**4c**) was isolated and characterized by MS−MS, along with 1D and 2D NMR spectroscopy (Figures S23 and S24). The NMR data clearly show the signals of the introduced POB side chain at δ 4.33, 5.75, 5.68, and 3.95 ppm ($CH_2CH{=}CHCH_2$), and the terminal alkynyl group at δ 4.05 and 2.80 ppm ($OCH_2C{\equiv}CH$). NOESY experiments (Figure S25) also show NOEs between the ArO−CH_2 protons of the POB side chain at 4.33 ppm and the C2 aryl singlet at 6.81 ppm, which indicates that isolation of solely *meta* alkylated DOPA peptides was possible.

Alkoxylation of Model Protein Substrates. To explore if the tyrosinase−COMT alkoxylation can be applied to protein substrates, we selected two acyl-carrier proteins (ACPs): End35 (from *Streptomyces fungicidicus*), which possesses four tyrosine residues,[60] and BtrI (from *Bacillus circulans*) which has no tyrosine residues.[61] End35 and BtrI are small (90 and 87 aa, respectively), share 25% sequence identity, and are predicted to adopt similar structures (Figures S43 and S44). In the case of BtrI, we engineered two variants, one with a tyrosine residue inserted after the N-terminal methionine (Y-BtrI) and another with a C-terminal tyrosine (BtrI-Y). Incubation of Y-BtrI and BtrI-Y with tyrosinase, for 30 min, and subsequent ESI-MS analysis revealed a new protein was formed. In both cases, the modified protein was 16 mass units higher, consistent with hydroxylation of the terminal tyrosine residue to DOPA (Y^{OH}-BtrI and BtrI-Y^{OH} see Figure 6). The relative intensity of the molecular ions in the ESI-MS is indicative of good conversions. When End35 was incubated with tyrosinase, under identical conditions to the BtrI experiments, we observed no modification of the tyrosine residues by ESI-MS. A structural homology model of End35 predicts that there could be at least 2 tyrosine residues on the protein surface (Figure S43). However, these residues are predicted to be present within α helices, and not more flexible loop regions. Overall, this suggests that tyrosine residues in more accessible and flexible terminal regions of proteins are more easily modified by tyrosinase. This is not surprising, given that tyrosinase enzymes are widely distributed across prokaryotes and eukaryotes.[48,49] If these enzymes were able to efficiently oxidize any surface tyrosine residue of a protein, then this would be detrimental to the producing organism, as the resulting *ortho*-quinone species could lead to nonspecific protein cross-linking and aggregation.[40−42] Incubation of Y-BtrI and BtrI-Y with tyrosinase, COMT, and AdoMet in tandem reactions led to selective methoxylation of the terminal tyrosine residues (Y^{OMe}-BtrI and BtrI-Y^{OMe}, see Figure 6). No methoxylation of End35 is evident under these conditions, suggesting again that terminal tyrosine residues are more easily modified. This may enable tyrosinase−COMT to selectively modify terminal tyrosine residues introduced into proteins that possess multiple tyrosine residues.

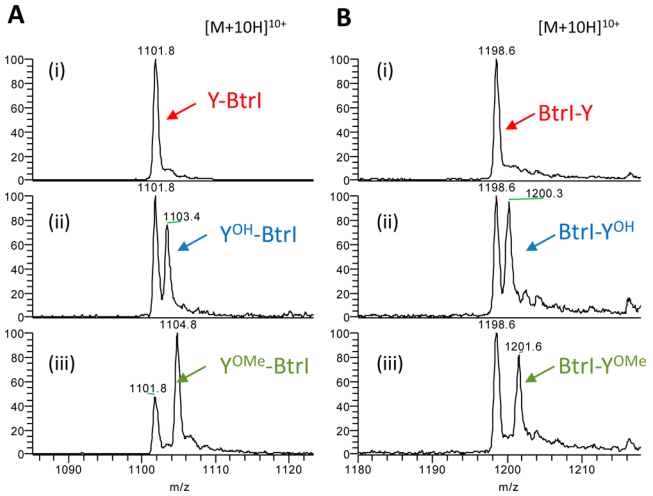

Figure 6. LC−ESI-MS data for protein modification. (A) The $[M + 10H]^{10+}$ molecular ions from hydroxylation and methoxylation reactions with Y-BtrI: (i) Y-BtrI, m/z 1101.8, has an observed deconvoluted average of mass 11 008.7 (calculated 11 009.4). (ii) Reaction with tyrosinase results in the formation of a new protein (Y^{OH}-BtrI) with m/z 1103.4 and a deconvoluted average mass of 11 024.2 (calculated 11 025.4). (iii) COMT-catalyzed methylation of Y^{OH}-BtrI gives Y^{OMe}-BtrI with m/z 1104.8 and a deconvoluted observed mass of 11 038.2 (calculated 11 039.4). (B) The $[M + 10H]^{10+}$ molecular ions of hydroxylation and methoxylation reactions with BtrI-Y: (i) BtrI-Y, m/z 1198.6, has an observed deconvoluted average mass of 11 976.9 (calculated 11 976.6). (ii) Reaction with tyrosinase results in the formation of new protein (BtrI-Y^{OH}) with m/z 1200.3 and a deconvoluted average mass of 11 992.9 (calculated 11 992.6). (iii) COMT-catalyzed methylation of BtrI-Y^{OH} gives BtrI-Y^{OMe} with m/z 1201.6 and a deconvoluted observed mass of 12 007.0 (calculated 12 006.6). Further protein MS data can be found in Supporting Information (Figures S45−S52).

CONCLUSION

In summary, we have developed a new approach for selective and bioorthogonal labeling of tyrosine residues in peptides with methyl and propargyl groups. Our method utilizes a tandem enzyme reaction comprising a fungal tyrosinase catalyzed hydroxylation of tyrosine to L-DOPA residues, followed by catechol-*O*-methyltransferase catalyzed *O*-alkylation. We have demonstrated the scope and versatility of tyrosinase−COMT alkoxylation with a number of tyrosine containing peptides: from linear synthetic peptides to human hormones and cyclic peptide antibiotics. In addition to accepting a range of L-DOPA peptide substrates, COMT is also able to accept the larger POB-bearing AdoMet analogue, facilitating the transfer of a bioorthogonal "handle". While tyrosinase−COMT can be used to derivatize tyrosine residues within more conformationally constrained cyclic peptides and internal positions in linear peptides, we found that the more flexible and accessible terminal tyrosine residues are most rapidly hydroxylated and alkylated. The promiscuity of COMT for AdoMet analogues also suggests this methodology is readily generalizable for the transfer of other functional groups.[15]

We have also demonstrated how tyrosinase−COMT can be used to selectively modify N- or C-terminal tyrosine residues in model protein substrates. Our approach is distinct from most

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other enzymatic methods that have been used to derivatize proteins which are largely based on natural post-translational modification (PTM) enzymes.[6−11,23−25] Typically, PTM enzymes require a specific peptide−substrate sequence or insertion of a peptide tag into a protein of interest for modification. Such tags can be large[6] and could alter the properties of the protein of interest. In contrast, tyrosinase and COMT have not evolved to modify specific peptide sequences and can modify tyrosine residues in a range of peptides and proteins provided the tyrosine is accessible to the enzymes. Our approach is also distinct from, and could potentially offer advantages over, nonenzymatic reactions that have been developed to modify tyrosine residues in peptides and proteins.[62−67] For example, tyrosine residues have been shown to undergo ene-type reactions,[62,63] palladium-mediated *O*-allylation,[64] Mannich-type reactions,[65] and coupling reactions with diazonium salts,[66] or with anilines in the presence of cerium(IV) ammonium nitrate.[67] These chemoselective reactions can give rise to mixtures of derivatized protein products when more than one surface tyrosine residue is present.[62−67] Modification of both tryptophan and tyrosine can also occur,[67] and in some cases, isomeric-adducts with different chemical linkages to the tyrosine residues are obtained.[67]

Further work will be necessary to assess in more detail the effects of peptide sequence and protein structure on tyrosinase−COMT alkoxylation reactions. For example, the selectivity with proteins possessing multiple tyrosine residues, including tyrosine residues within flexible terminal or loop regions as well as more structured motifs needs to be evaluated. Nevertheless, the results presented here suggest tyrosinase−COMT could be further developed for the selective functionalization of proteins. Moreover, the fact that the tyrosinase−COMT method is distinct from existing approaches (based on PTM enzymes or chemoselective chemistries) provides the possibility that new selectivity for protein modification may be developed. For instance, we recently showed how the regioselectivity of COMT can be altered, with smaller catechol substrates and AdoMet analogues, through active site mutagenesis and changes in quaternary structure.[47] Through similar protein engineering approaches, it may be possible to improve the activity and selectivity of tyrosinase or COMT for peptide and protein substrates.

Finally, the methodology described here might also be used to label or capture naturally occurring proteins or peptides with L-DOPA residues. In humans, DOPA-containing proteins have been implicated in neurodegenerative disorders and other diseases associated with aging.[34−40,68] However, DOPA incorporation typically occurs at low levels, which makes isolation and analysis of DOPA proteins, or their products of proteolysis, challenging. Therefore, labeling or capturing human DOPA-containing proteins and peptides, using COMT or improved engineered COMT variants, may be useful for biomedical diagnostic applications.

MATERIALS AND METHODS

Hydroxylation of Peptides. Hydroxylation assays were conducted with $MgCl_2$ (3 mM), mushroom tyrosinase (σ 0.2 μM), ascorbic acid (12.5 mM), and peptide substrate (0.5 mM), in a 20 mM potassium phosphate buffer pH 7.4. The assay mixtures were incubated at 30 °C with 800 rpm agitation, for 15 min. Termination of the reaction was achieved with 1% formic acid (0.1% final concentration), and the samples were then centrifuged to remove proteins (13 000*g*, room temperature, 10 min). All assays were analyzed by MALDI-TOF-MS on a Bruker Ultraflex in reflector mode using DHB as matrix. Selected products were further characterized by MALDI-TOF-MS/MS on a Bruker Ultraflex in LIFT mode using DHB as matrix. The percent conversions were determined by analytical HPLC for synthetic peptides, KNYLDF and KNFLDY, using an Agilent 1260 Infinity HPLC and Phenomenex Kinetex C_{18} 5 μm, 4.6 × 150 mm column; flow rate 1 mL/min; wavelength 280 nm; gradient of 0−2 min 5% B, 2−7 min 5−75% B, 7−7.1 min 75−95% B, 7.1−9 min 95% B, 9−9.1 min 95−5% B, 9.1−12 min 5% B; mobile phase A, H_2O and 0.1% TFA; mobile phase B, acetonitrile 0.1% TFA. In contrast, percent conversions were determined for goserelin and oxytocin by the following method: Agilent 1260 Infinity HPLC and Phenomenex Kinetex C_{18} 5 μm, 4.6 × 150 mm column; flow rate 1 mL/min; wavelength 280 nm; gradient of 0−2 min 5% B, 2−9 min 5−65% B, 9−10.1 min 65−95% B, 10.1−11 min 95% B, 11−11.1 min 95−5% B, 11.1−14 min 5% B; mobile phase A, H_2O and 0.1% TFA; mobile phase B, acetonitrile 0.1% TFA. For KNYLDY, the percent conversions were determined using an Agilent 1260 Infinity HPLC and Phenomenex Kinetex C_{18} 5 μm, 4.6 × 150 mm column, with a flow rate of 1 mL/min; wavelength of 280 nm; and a gradient of 0−2 min 5% B, 2−10 min 5−20% B, 10−10.1 min 20−95% B, 10.1−12 min 95% B, 12−12.1 min 95−5% B, 12.1−14 min 5% B; mobile phase A, H_2O and 0.1% TFA; mobile phase B, acetonitrile 0.1% TFA.

Methoxylation of Peptides. Methoxylation assays were conducted with $MgCl_2$ (3 mM), mushroom tyrosinase (0.2 μM), ascorbic acid (12.5 mM), COMT (15 μM), AdoMet (2 mM), and peptide substrate (0.5 mM), in a 20 mM potassium phosphate buffer pH 7.4. The assay mixtures were incubated at 30 °C with 800 rpm agitation, for 5 h. Reactions were terminated and analyzed by HPLC, MS, and MS−MS as described above.

Alkylation of DOPA-Containing Peptides. To phosphate buffer were added the following to make up the assay mix: $MgCl_2$ (3 mM), COMT (50 μM), AdoMet (2 mM), hydroxylated peptide substrate (0.5 mM). Assays were carried out, with shaking (850 rpm) at 37 °C for 1 h. The POB-AdoMet reactions were carried out with $MgCl_2$ (3 mM), COMT (5 μM), POB-AdoMet (2 mM), hydroxylated peptide substrate (0.5 mM). Assays were left shaking (850 rpm) overnight at 37 °C. Reactions were terminated and analyzed by HPLC, MS, and MS−MS as described above.

Modification of Terminal Tyrosine Residues of Acyl Carrier Proteins. The ACP variants, Y-BtrI and BtrI-Y, were engineered as described in the Supporting Information. Hydroxylation of Y-BtrI and BtrI-Y was carried out by incubating the ACP substrates (40 μM) with mushroom tyrosinase (0.2 μM), ascorbic acid (12.5 mM), $MgCl_2$ (3 mM), in 20 mM potassium phosphate buffer pH 7.4, at 30 °C with agitation (850 rpm), for 30 min. Methoxylation of Y-BtrI and BtrI-Y was achieved in tandem enzyme assays with $MgCl_2$ (3 mM), mushroom tyrosinase (0.2 μM), ascorbic acid (12.5 mM), COMT (15 μM), AdoMet (2 mM), protein substrate (40 μM), in a 20 mM potassium phosphate buffer pH 7.4 at 30 °C with agitation (850 rpm), for 3 h. Reaction mixtures, from both hydroxylation and methoxylation assays, were then passed through a 50 kDa MWCO-filter and buffer exchanged into 50 mM ammonium acetate, pH 7.5, prior to analysis by mass spectrometry.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b10928.

Additional experimental details including methods for overproduction and purification of proteins (COMT and ACPs), synthesis of peptide substrates and POB-AdoMet, as well as method for isolation and characterization of the products of enzymatic reactions; supplementary figures which describe the MS, MS−MS sequencing, and NMR data used in the characterization of the enzymatic reaction products (PDF)

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AUTHOR INFORMATION

Corresponding Author

*jason.micklefield@manchester.ac.uk

Author Contributions

†A.-W.S. and M.R.B. contributed equally to this work.

Notes

The authors declare no competing financial interest.

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