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Site-Specific Protein Modification with a Dirhodium Metallopeptide Catalyst

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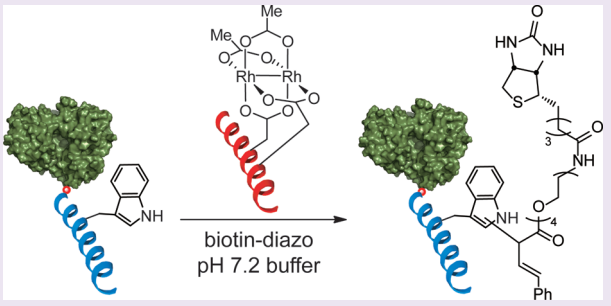
Supporting Information

ABSTRACT: A new method for chemical protein modification is presented utilizing a dirhodium metallopeptide catalyst. The combination of peptide-based molecular recognition and a dirhodium catalyst with broad side-chain scope enables site-specific protein functionalization. The scope and utility of dirhodium-catalyzed biomolecule modification is expanded to allow reaction at physiological pH and in biologically relevant buffer solutions. Specific protein modification is possible directly in *E. coli* lysate, demonstrating the remarkable activity and specificity of the designed metallopeptide catalyst. Furthermore, a new biotin-diazo conjugate **1b** is presented that allows affinity tagging of target proteins.



Proteins contain diverse and complex primary structure. Post-translational chemical modification of these polyfunctional structures is an important goal of chemical biology that enables the study of protein function, protein−protein interactions, and time-resolved localization in living cells. In addition, chemical modification is emerging as an important way to improve efficacy and pharmacokinetic properties of recombinant protein therapeutics.[1,2]

The development of selective methods for protein functionalization is an active and rapidly evolving field.[3−7] Most chemical methods rely on residue-selective capabilities, such as nucleophilic cysteine or lysine side chains. Because many copies of each amino acid are present in a typical protein, it is difficult to produce homogeneous functionalized protein and often impossible to modify a specific protein in a complex mixture. To impart true single-site reactivity, modern methods incorporate a non-natural amino acid with orthogonal reactivity for reaction with a functional reagent. Important examples of these processes include amino acids containing azide, ketone, or halide functional groups.[8,9] In addition, recombinant methods are used to incorporate "tag" sequences that later react in a sequence-specific manner to produce a covalently modified protein.[6,7,10] The use of tags can be grouped into two categories: sequences or domains that react stoichiometrically with small-molecule reagents,[11−14] such as arsine-based FlAsH probes[15,16] or covalent TMP-binding domains,[17,18] and sequences targeted by enzymatic reactions,[19] such as formylglycine generation,[1] biotin ligase,[20,21] or phosphopantetheinyl transferase.[22−25] Although useful tools, concerns about robustness under harsh conditions, generality across proteins and expression organisms, and scalability may limit the effectiveness of these methods. In this paper, we describe a site-specific protein functionalization catalyzed by dirhodium metallopeptide that allows biotin incorporation at a specific tryptophan residue.

Our approach integrates molecular recognition with a transition-metal catalyst in order to realize precise site-specificity on the basis of molecular shape rather than inherent chemical reactivity (Figure 1). When we began our studies of dirhodium metallopeptides, it was known that the complex $Rh_2(OAc)_4$ could catalyze protein modification at tryptophan residues.[3,26,27] With peptide models, we subsequently demonstrated that transient assembly of a designed metallopeptide with the target sequence produced rate enhancements of $\geq 10^3$ and expanded the amino acid scope of dirhodium-catalyzed modification to include amino acids (see Figure 1) comprising more than 50% of the amino acid makeup of natural proteins, with little to no background reactivity.[28,29] In this work, we sought to extend these initial studies on model peptide substrates in controlled environments to examine the reactivity of whole proteins in a complex, cell-like environment. In addition, we aimed to incorporate a useful chemical handle by synthesizing a functionalized diazo reagent. Because a dirhodium metallopeptide method offers a catalytic, yet chemical, means of protein functionalization, it occupies a middle ground between methods based on chemical reagents and those based on catalytic enzyme function, and may exhibit benefits of both chemical and enzymatic approaches.

RESULTS AND DISCUSSION

Previous work with dirhodium catalysis in protein[3,26,27] and peptide[28,29] modification raised a number of limitations to

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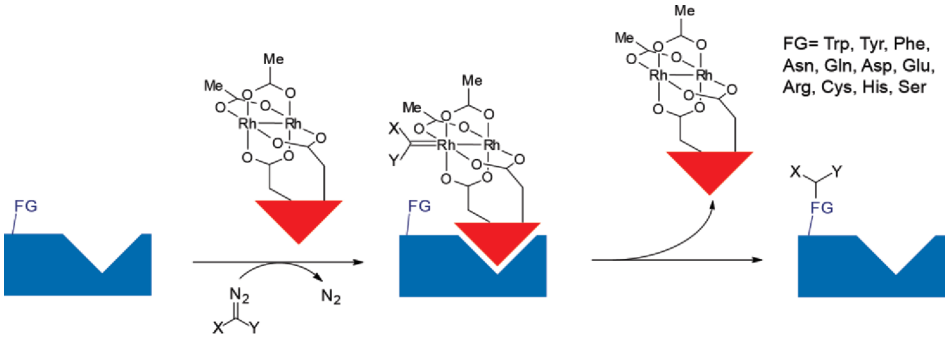

Figure 1. Conceptual depiction of the proximity-driven mechanism resulted from the combination of molecular recognition and promiscuous dirhodium catalysis. Dirhodium metallopeptides are capable of functionalizing >50% of the amino acid makeup of natural proteins.

address before proximity-driven catalysis could be a general tool for protein functionalization. First, previous work has employed only simple diazo reagents without functional handles, a necessity for many applications.[30] In addition, low pH (≤6.2) and a special hydroxylamine buffer environment have been required for reasonable reactivity, limiting the generality of the method and its usefulness in biological environments. Extensive optimization by Francis[3,26,27] determined that *N-tert*-butylhydroxylamine·HCl (TBHA) was a unique buffer that allowed tryptophan modification to proceed at pH 6.2 or below with useful efficiency. More typical and biologically relevant buffers, such as PBS, resulted in significantly lower efficiency.

Fortunately, proximity-driven catalysis addresses these limitations. For orienting studies we examined heterodimerization of designed coiled coils, studied previously by our group,[28,29] as a model protein−peptide interaction to template modification (Figure 2). The metallopeptide $K3_{a,e}Rh_2$ and its complementary substrate peptide, $E3_gW$, were designed based on the well-known E3/K3 heterodimeric pair,[31] with specific amino-acid substitutions at the interfacial residues to position a dirhodium catalyst near a tryptophan residue, selected because it exhibits the fastest modification rate among the 11 reactive amino acids in proximity-driven dirhodium modification.[29] As shown in Table 1, coil-templated catalysis allows efficient modification even at biological pH (entry 2). Furthermore, many other common buffers are compatible with the modification reactions, allowing >90% conversion within a few hours. TBHA remains the optimal buffer, providing >90% conversion in under 45 min, and the increased reaction rate in TBHA buffer may be beneficial for some applications. These experiments validate the use of dirhodium metallopeptides for modification in biologically relevant pH and buffer.

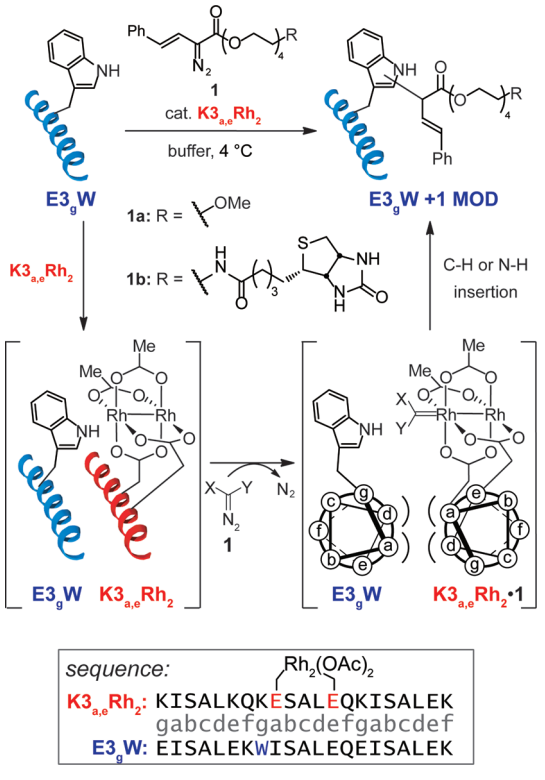

Figure 2. Proximity-driven modification by transient coiled-coil assembly. See Table 1 for results.

To address the need for a diazo reagent that would facilitate the introduction of a unique chemical handle, we synthesized a bifunctional biotin-diazo affinity tag, **1b** (Scheme 1). From nonsymmetrical PEG **3**,[32−34] we attached a biotin molecule *via* its NHS ester, esterified the free alcohol with styrylacetic acid, and performed diazo transfer with *p*-acetamidobenzenesulfonyl

Table 1. Buffer Scope for Metallopeptide-Catalyzed Modification of $E3_gW$[a]

			convn (%)[d]	
entry	buffer[b]	pH[c]	45 min	4 h
1	TBHA	6.2	93	>95
2		7.4	90	>95
3	PBS	7.4	80	>95
4	TES	7.4	55	84
5	MOPS	7.4	76	>95
6	PIPES	7.6	66	86
7	HEPES	7.5	43	49

[a] Reaction conditions: $E3_gW$ (50 μM), $K3_{a,e}Rh_2$ (5 μM), diazo **1a** (3.5 mM), 0.1 M aq buffer with 2% *t*-BuOH. [b] Buffer abbreviations: TBHA = *tert*-butylhydroxylamine, PBS = phosphate-buffered saline (0.0067 M PO_4^{2-}), TES = 2-[[1,3-dihydroxy-2-(hydroxymethyl) propan-2-yl]amino]ethanesulfonic acid, MOPS = 3-morpholinopropane-1-sulfonic acid, PIPES = 1,4-piperazinediethanesulfonic acid, HEPES = 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid. [c] Measured at 25 °C (±0.1), [d] Conversion based on MALDI-TOF MS. Validation of MS conversion data by HPLC for E3 modification has been reported previously.[28]

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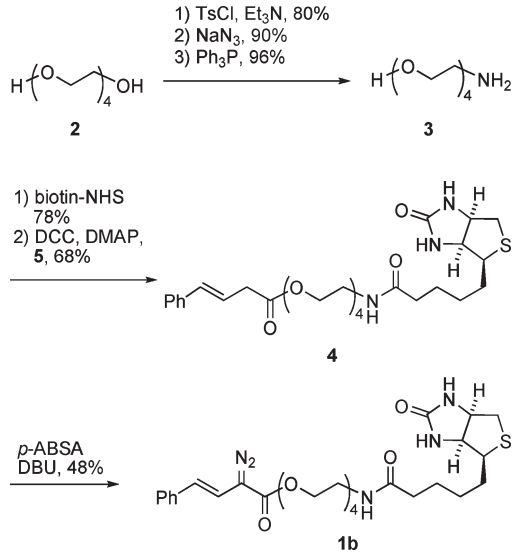
Scheme 1. Synthesis of a Biotin-diazo Reagent[a]



[a] **5** = *trans*-styrylacetic acid; p-ABSA = *p*-acetamidobenzenesulfonyl azide.

azide (*p*-ABSA) to afford the bifunctional reagent **1b**. Like the simple styrylacetic diazo derivatives we have used previously, reagent **1b** must be handled with care but is stable to silica gel chromatography, and stock solutions in *tert*-butanol can be stored for weeks in the dark at −20 °C.

Gratifyingly, biotin-diazo **1b** exhibits reactivity comparable to that of the simple diazo reagent **1a**. We first examined this reactivity in the context of peptide modification based on a coiled-coil model, as studied previously[28,29] The reaction was monitored by MALDI-TOF MS. After 16 h, the singly and doubly biotinylated E3$_g$W peptides were detected (Figure 3a, top spectrum). Over 85% of the starting peptide was modified upon treatment with 2.5 μM metallopeptide and 750 μM biotin-diazo (**1b**) in PBS buffer (pH 7.2). In sharp contrast, $Rh_2(OAc)_4$ showed poor reactivity even with much higher catalyst loading (25 μM, <10% conversion). The site of the modification was unambiguously determined by MS/MS analysis (Figure 3b). Fragmentation of the product parent ion (m/z 3119.6) provided an ion (m/z 720.3) derived from cleavage of the modified tryptophan side chain, as well as expected ladder peaks including the y13 and y14 fragments immediately before and after the modified W9 residue (Figure 3).

With a method in hand that allows site-specific biotinylation in physiological buffer, we moved to modification of a large protein directly in cell lysate. We chose to employ the same coiled-coil recognition used in the studies described above. Recombinant maltose binding protein (MBP) was expressed in *E. coli* as a fusion with the 21-amino-acid coil, E3$_g$W, at the C-terminus. An enterokinase cleavage site[35] was included between the two fused domains. After expression of the MBP-E3$_g$W fusion, the lysate was subjected to metallopeptide-catalyzed biotinylation with K3$_{a,e}$Rh$_2$, and the reaction was analyzed by SDS-PAGE (Figure 4a). As expected, a single band in Western blot analysis demonstrated highly selective modification of the target MBP-E3$_g$W. Nonselective protein modification was not observed, and control experiments without dirhodium or with the small-molecule catalyst $Rh_2(OAc)_4$ provided no modification. In

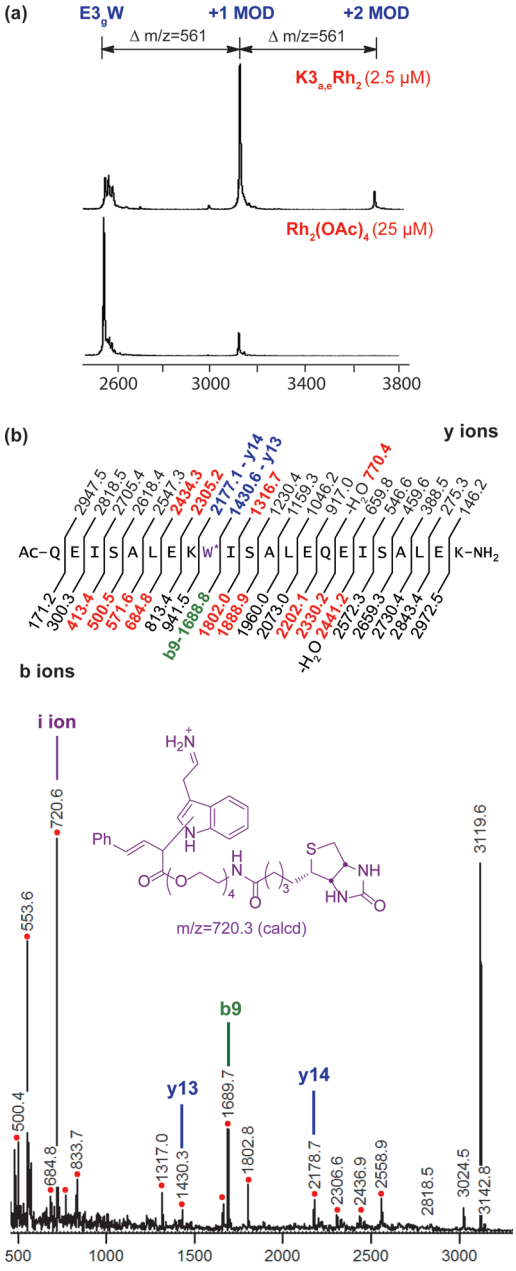


Figure 3. (a) MALDI-TOF mass spectra demonstrating biotinylation of E3$_g$W catalyzed by 2.5 μM K3$_{a,e}$Rh$_2$ (top) and with 25 μM $Rh_2(OAc)_4$ (bottom) after 16 h. The observed increase is consistent with calculated mass. Reaction condition: 25 μM E3$_g$W, 2.5 μM K3$_{a,e}$Rh$_2$, or 25 μM $Rh_2(OAc)_4$, 750 μM biotin-diazo **4**, 0.1 M PBS buffer (pH 7.2) at 4 °C for 16 h. (b) MS/MS analysis of singly biotinylated E3$_g$W parent ion (m/z = 3119.6). Peaks matching predicted fragments are labeled with a red dot. The largest peak (m/z 720.6) corresponds to side-chain cleavage of biotinylated tryptophan.

addition, MBP without the E3$_g$W tag showed no evidence of biotinylation. The enterokinase cleavage site enabled us to cleave the biotin-containing fragment prior to Western analysis. After enterokinase treatment, only traces of biotinylation were imaged by Western blot, identifying the E3$_g$W helix as the site of modification. A dose-dependent Western blot was used to estimate conversion of the biotinylation reaction relative to a

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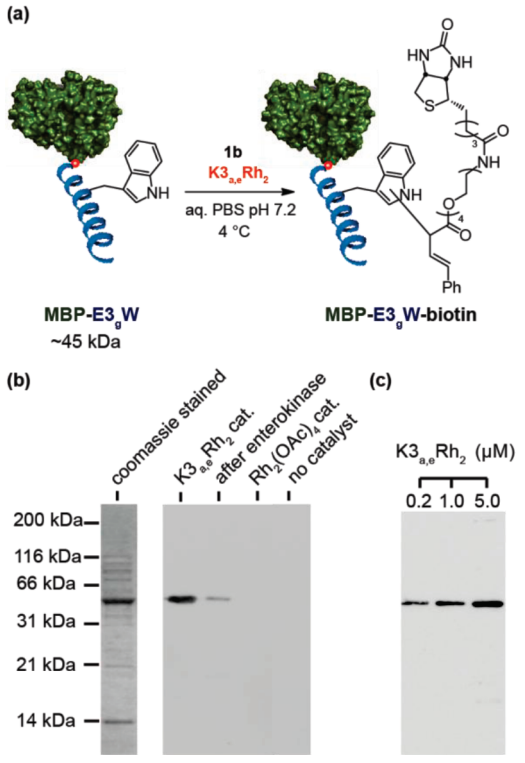

Figure 4. (a) Selective biotinylation of MBP-E3$_g$W using complementary metallopeptide catalyst. Enterokinase cleavage site is indicated by small red circle. Modification conditions: 1 μM MBP-E3$_g$W, 0.2−5 μM K3$_{a,e}$Rh$_2$, or 10 μM Rh$_2$(OAc)$_4$, 100 μM biotin-diazo (**1b**) in PBS buffer (pH 7.2, 0.1 M), 4 °C, 16 h. (b) Coomassie stained gel of cell lysate (left) and biotin-specific Western analysis image (right) of lane 1, biotinylated lysate; lane 2, enterokinase-treated biotinylated lysate; lane 3, modification reaction of cell lysate catalyzed by Rh$_2$(OAc)$_4$; lane 4, mixture of cell lysate and biotin-diazo (**1b**) (no catalyst). (c) Western analysis of reaction mixtures catalyzed by 0.2, 1.0, and 5.0 μM dirhodium metallopeptide. The corresponding modification ratios were quantified as 5%, 20%, and 70% based on densitometric comparison with authentic monobiotinylated horseradish peroxidase standards.

biotinylated protein standard. Metallopeptide concentrations ranging from 0.2 to 5.0 μM afforded estimated conversions of 5−70%, in line with that expected for a dose-dependent catalyst. The modification could also be analyzed by MALDI-MS for reactions run on purified MBP-E3$_g$W (Figure S1), but we have had difficulty obtaining MALDI-MS data on crude lysate mixtures for even unmodified MBP-E3$_g$W.

The lack of lysozyme modification in lysate reactions is significant. No evidence of biotinylation is observed for the lysozyme band at 14 kDa (Figure 4b, Coomassie stain). However, lysozyme contains a tryptophan residue and is one of the proteins that has been previously shown to react in nonselective tryptophan modification catalyzed by dirhodium (Rh$_2$(OAc)$_4$).[3,26] Nondirected tryptophan modification with Rh$_2$(OAc)$_4$ does require more forcing conditions (higher temperature, lower pH, increased concentration), and the milder, more dilute conditions—made possible by molecular recognition in our metallopeptide catalyst—enable MBP-E3$_g$W biotinylation with complete specificity.

Conclusion. This work significantly expands the capabilities of proximity-driven metallopeptide catalysis for the functionalization of biomolecules, including the first dirhodium-catalyzed functionalization of protein targets in lysate. The pH and buffer scope of dirhodium reactivity is greatly expanded in reactions templated by molecular recognition. It is now possible to functionalize protein targets at physiological pH and in diverse buffers, including PBS. Furthermore, a bifunctional biotin-diazo reagent **1b** was developed for installing a useful affinity handle.

The principles of proximity-driven protein modification, explored here in the context of recombinant MBP, should be applicable to a broad range of recombinant or natural proteins. Although this initial report uses recombinant methods to install a designed molecular recognition motif, targeting natural proteins on the basis of binding interactions, such as protein−protein or protein−peptide interactions, should be possible. This capability would separate the present method from other methods for specific protein functionalization, which require the presence of recombinant tags. Specific chemical modification of natural proteins remains an important unsolved problem. However, we have previously reported that the coiled-coil approach outlined here is amenable to targeting natural coils,[29] and recent work in our laboratory (unpublished) demonstrates that the protein modification concept can be extended to other natural protein structures that exhibit protein-peptide interactions.

The method described here is also a significant step toward the development of chemical catalysts that rival the selectivity of natural metalloenzymes.[36,37] Combining molecular recognition with metallocarbene reactivity with broad side-chain scope enables modification of protein target on the basis of molecular shape rather than inherent reactivity normally associated with chemical catalysts. Developing chemical catalysts for biomolecule substrates that override inherent reactivity has been a challenging problem.[38−41] In modifying a specific protein in cell lysate, we have created a metallopeptide capable of catalyzing a chemical reaction at a specific natural amino acid side chain in a remarkably complex milieu.

METHODS

Chemical Synthesis. Peptides were synthesized with an AAPPTEC APEX 396 Automated Multipeptide Synthesizer using standard solid-phase Fmoc protocols.[42,43] The catalyst K3$_{a,e}$Rh$_2$, reported previously,[28] was synthesized by direct metalation of the peptide with the heteroleptic dirhodium complex, Rh$_2$(OAc)$_2$(tfa)$_2$, according to established protocols.[43,44] The diazo reagent **1a** was produced as previously described.[28] Experimental details and characterization data for the preparation of diazo reagent **1b** are provided in Supporting Information.

Protein Expression and Purification. *E. coli* cells (BL21, Rosetta) transformed with the MBP-E3$_g$W plasmid (purchased from Genscript, see Supporting Information for sequence and details) were grown in LB (5 mL) with ampicillin (50 μg mL^{-1}) at 37 °C to OD$_{600}$ ~1 and induced with IPTG (0.75 mM). Cells were grown for 3 h at 37 °C to allow for expression. Cell paste was resuspended in PBS buffer (500 μL, pH 8.0) containing MgCl$_2$ (1 mM), lysozyme (500 μg/mL), and DNase I (4 units mL^{-1}). The cells were lysed by freeze−thaw (−80 °C) overnight, and the cell debris was separated by centrifuge. The crude lysate was used directly in modification reactions.

Protein Modification. Metallopeptide K3$_{a,e}$Rh$_2$ was dissolved in water (100 μM). A stock solution of diazo reagent **1b** in 5% *t*-BuOH/H$_2$O (2.0 μM) was also prepared. MBP-E3$_g$W crude lysate (5.0 μL, 1.0 μM final concn) was added to the aqueous buffer, followed by K3$_{a,e}$Rh$_2$ solution (0.5 μL, 5 μM final concn). The reaction was initiated by addition of diazo stock solution (0.5 μL, 100 μM final concn). The total reaction volume was 10 μL with 0.25% *t*-BuOH cosolvent. The

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reaction tube was initially mixed for ca. 30 s with a benchtop vortex mixer and was then placed in a bed shaker in a cold room (4 °C) for 16 h. The crude reaction mixture was used directly in SDS−PAGE Western blot analysis. See Supporting Information for details.

■ ASSOCIATED CONTENT

Supporting Information. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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