

A protein functionalization platform based on selective reactions at methionine residues

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Nature displays a remarkable ability to carry out site-selective post-translational modification of proteins, therefore enabling a dramatic increase in their functional diversity¹. Inspired by this, chemical tools have evolved for the synthetic manipulation of protein structure and function, and have become essential to the continued advancement of chemical biology, molecular biology and medicine. However, the number of chemical transformations suitable for effective protein functionalization is limited because the stringent demands inherent to biological systems preclude the applicability of many potential processes². Put simply, these chemical transformations often need to be selective at a single site on a protein, proceed with very fast reaction rates, operate under biologically ambient conditions and should provide homogeneous products with near perfect conversion²⁻⁷. While many elegant bioconjugation methods exist at cysteine, lysine and tyrosine, we reasoned that a method targeting a less explored amino acid would significantly expand the protein functionalization toolbox. Herein, we report the development of a multifaceted-approach to protein functionalization based on chemoselective labelling at methionine residues. By exploiting the unique electrophilic reactivity of a bespoke hypervalent iodine reagent, one can target the *S*-Me group in the side-chain of methionine. The bioconjugation reaction is fast, selective, operates at low μM concentrations and is complementary to existing bioconjugation strategies. Moreover, the new reaction produces a protein conjugate that is, itself, a high energy intermediate with reactive properties that can serve as a platform for the development of secondary, visible-light mediated bioorthogonal protein functionalization processes. Taken together, the merger of these approaches provides a versatile platform for the development of distinct transformations that can deliver versatile, information-rich protein conjugates directly from the native biomacromolecules.

The sheer structural diversity of the proteome in any single organism means that no one protein functionalization method is likely to provide a universal solution for the preparation of protein constructs (Fig. 1a)⁸⁻¹¹. Although encoded by the AUG start codon at the beginning of protein synthesis, methionine is often post-translationally excised and thus has a low abundance in proteins (around 2%). It is also frequently used as a replacement for hydrocarbon-containing residues¹². The limited function of methionine compared to other residues, being mainly responsible for protection against oxidative stress, means that its functionalization is less likely to impair protein function¹³. Targeting Met would not only provide a distinct bioconjugation approach but also, using our strategy, the resulting Met conjugates provide exploitable intrinsic reactivity such that they could lead to the rapid synthesis of diverse, functional protein constructs from native proteins.

To date, the only effective method for bioconjugation at methionine was recently reported by Chang et al, who showed that the oxidation of thioethers with oxaziridine reagents provided the basis for an elegant bio-inspired strategy to form stable protein-bound sulfoximines¹⁴. We aimed to target the polarizable thioether on the Met side chain with a suitable electrophile to form a cationic sulfonium species, selectively installing a versatile payload and distinct functionality at a methionine residue, thereby providing a fundamentally different bioconjugation approach (Figure 1a)⁸⁻¹¹. Methionine residues react with cyanogen bromide (CNBr), however, such a process cannot function as a bioconjugation method because the reactivity of the resulting unstable cyano-substituted sulfonium cation triggers protein backbone cleavage¹⁵. Conversely, methionine residues undergo slow S-alkylation reactions using iodoacetamide or with other benzyl-derived electrophiles to form relatively stable trialkyl sulfonium cations¹⁶⁻¹⁹. Striking a balance between the stability of the protein-bound sulfonium cations, compatibility of reaction conditions and the reaction rate of the thioether with suitable electrophiles has, to date, precluded the development of an effective alkylation-based method for bioconjugation at methionine. Guided by these limitations, we posited that a distinct class of electrophile, based on the hypervalent iodine scaffold (λ -3-iodanes)²⁰, could make for a functioning bioconjugation process at

methionine. Tailoring the substituents and counteranion on the I(III) atom should allow tuning of the reactivity of the polarizable I(III) nodal center to dovetail with the electron lone pair of the thioether and, also, the stability of the resulting sulfonium conjugate through modulation of the electronic features of the groups directly attached to the cationic sulfur motif. We had noted that a structurally remarkable iodonium salt (**1** in Figure 1, R=Et and X=OTf) reacts rapidly with dimethylsulfide (the simplest possible mimic of the thioether motif in methionine) to form a sulfonium adduct^{21,22}. Successful reaction of this iodonium salt with methionine would not only represent a distinct method for bioconjugation, but also deliver a high-energy conjugate equipped with reactive ‘on protein’ groups that could serve as a basis for designing new transformations towards diversely-functionalized protein constructs (Figure 1b).

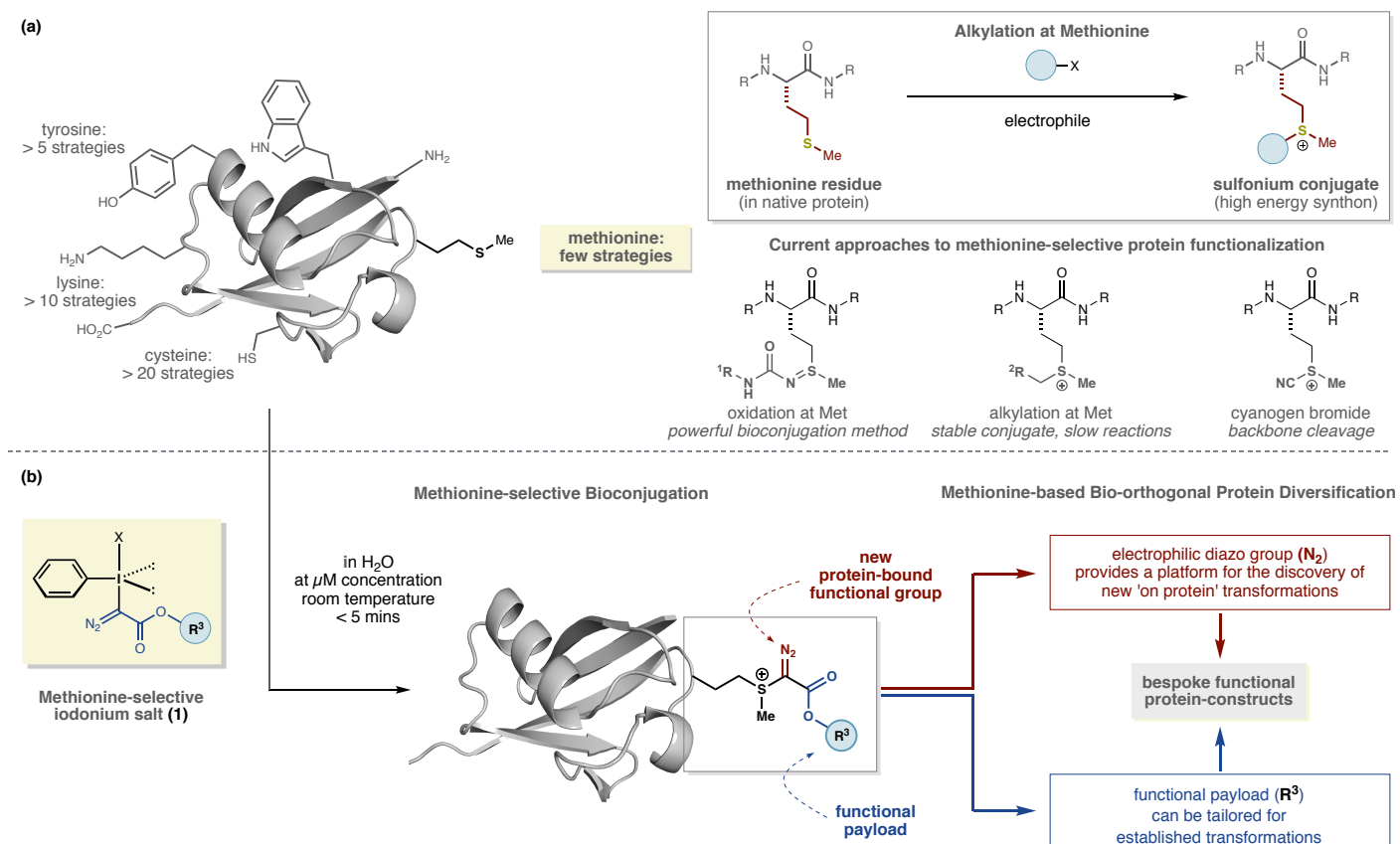


Figure 1. The evolution of a methionine-selective protein functionalization strategy (a) Existing protein functionalization tactics, and the potential for methionine-selective bioconjugation; R = peptide or protein, R¹ = various organic groups, R² = aryl or ester

group (**b**) Functionalized hypervalent iodine reagents enable methionine-selective protein modification, leading to methionine-based bioorthogonal protein diversification. X = leaving group; R³ = functional payload.

We first examined the reaction between dipeptide **2a** and iodonium triflate **1a** (Figure 2a). We observed the formation of the desired sulfonium-conjugate **3a**, though it was clear from the low yield (27%) that the iodonium salt displayed poor stability in aqueous solution. By tailoring the aryl group of the iodonium salt (to the electron-deficient 2,4-difluorobenzene) and replacing the triflate counteranion with tetrafluoroborate (**1b**), we found that a readily prepared reagent **1b** displayed superior physical properties (half-life in water is > 50 h). Treatment of reagent **1b** with dipeptide **2a** gave 70% of the desired product **3b** accompanied by the corresponding sulfoxide (not shown) after reaction for 30 minutes.

Moving to a more complex substrate, the GLP-1 receptor agonist, exenatide (Byetta™, **2b**, a 39-residue helical polypeptide containing a single mid-chain methionine, Figure 2b), we found that treatment of a 100 μM aqueous solution with **1b** led to decomposition of the polypeptide. A key breakthrough revealed that the addition of a low concentration of thiourea (20 mM) resulted in a dramatic improvement of the reaction, such that sulfonium-conjugate **4a** was formed with 68% conversion in less than 2 minutes, accompanied by non-specific oxidation and labelling. Further improvements could be made by running the reaction in the presence of TEMPO (10 mM), which minimized formation of oxidative by-products. Addition of aqueous formic acid solution (5 mM, approximately pH 3) reduced the non-specific labelling by-products to trace levels. Finally, we found that the labelling process proceeded effectively when conducted in distilled water. Routine analysis of ESI-MS and MS-MS fragmentation data confirmed selective reaction at methionine. Although the concentration of thiourea is well below the levels needed for protein denaturation, we were pleased to observe that the CD spectra of exenatide-conjugate **4a** confirmed retention of the characteristic helical structure (See the supplementary information S35 for details). While we are not yet certain of the remarkable role of thiourea,

it is important to note its presence appears to be fundamental in providing a bioconjugation process at reaction rates needed for transformation on complex biomolecules. With these refined conditions, the thiourea-accelerated bioconjugation was almost instantaneous at exenatide concentrations ranging from 5-500 μM without compromising the conversion, and a larger, milligram-scale reaction enabled purification of conjugate **4a** by semi-preparative HPLC to give 79% yield of isolated product. Given the similarity of the exenatide conjugate to the intermediate that would arise from reaction of the polypeptide with cyanogen bromide (leading to peptide cleavage), it is remarkable that **4a** has a half-life in water of over 100 hours. In comparison to the corresponding reaction of methionine residues with cyanogen bromide, we believe that the observed stability of **4a** is a result of the ethyl diazoacetate motif imparting a lesser electron-withdrawing effect on the sulfonium salt (than the cyano-group), which in turn leads to a species that is less reactive to attack by the proximal carbonyl group and is, hence, a more stable conjugate.

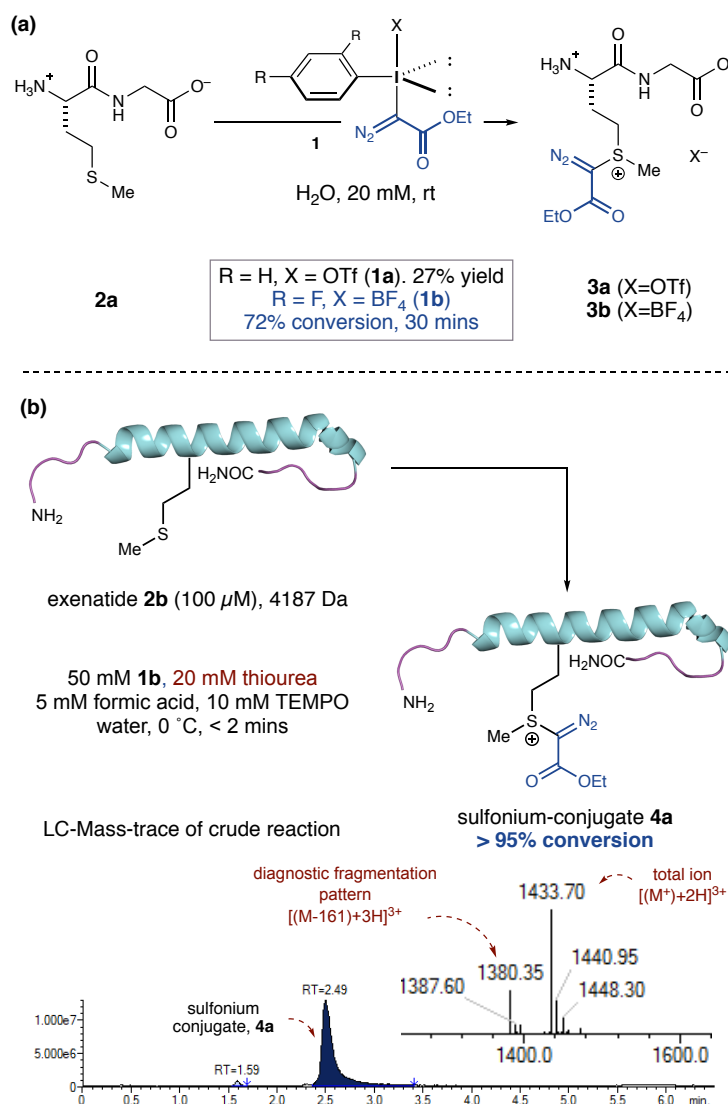


Figure 2. Evolution of a methionine-selective bioconjugation strategy. **(a)** Initial results for functionalization at methionine with hypervalent iodine reagents. **(b)** Optimal process for the thiourea-accelerated methionine selective bioconjugation of exenatide.

The optimized conditions, using **1b**, were used to evaluate the substrate scope (Figure 3a). Random coil polypeptides that contain methionine, such as AviptadilTM and tetracosactide, are efficiently converted to the sulfonium-conjugates **5** and **6** respectively. GTP-binding protein fragment-G α , which contains a free sulfhydryl group in a N-terminal cysteine residue, underwent smooth methionine-labelling to **7** with concomitant oxidative disulfide formation. Teriparatide, a polypeptide containing two methionines, was converted to its bis-sulfonium conjugate (**8**) with good conversions. α -Lactalbumin, a globular protein

displaying a readily oxidized methionine residue, undergoes bioconjugation to **9** with minimal competitive oxidation. Aprotinin (Trasylol) also forms the corresponding sulfonium-conjugate **10** in good yield. Particularly significant is the observation that two cysteine-disulfide linkages within the structure of aprotinin (and the labelling to **7**) are not affected by **1b**, with a single methionine-derived conjugate obtained in high conversion. When the methionine residues are buried within the tertiary structure of the protein, for example with RNA-ase B, the bioconjugation did not occur, highlighting an inherent selectivity for the labeling process between exposed and inaccessible thioether groups. This feature is highlighted by the case of ubiquitin; the *N*-terminal methionine residue has only moderate surface exposure and could slow down the rate of functionalization to the point where oxidation becomes competitive. Accordingly, reaction under de-oxygenated conditions allowed for efficient conversion to the labeled product (85%), with a 10:1 label:oxidation ratio (**11**). The methionine-selective bioconjugation strategy effectively functionalizes a range of polypeptides and proteins in high conversion at μM concentrations and in very short reaction times. Given the reactive appearance of **1b** – a carbon-electrophile displaying two of the best leaving groups known to organic chemists – it is remarkable that this species selectively engages the moderately nucleophilic methionine residue in the presence of competitively nucleophilic and oxidizable amino acid residues. Bioconjugation strategies that target other amino acids should, therefore, be compatible with, and complementary to, our methionine-functionalization process. To exemplify this, we showed that the GTP-binding protein fragment- $G\alpha$ could be first labelled at cysteine, using a maleimide derivative,²³ and then conjugated at methionine using iodonium salt **1b** to form **12**. The methionine-selective process does not interfere with the cysteine-maleimide motif, which itself contains a thioether linkage, thereby highlighting possible applications towards multi-site heterolabelling of proteins²⁴.

The modular synthesis of the hypervalent iodine reagents enables facile incorporation of different acyl groups attached to the diazo motif, allowing the transfer of a range of functional payloads to proteins (Figure 3b).

Functional groups relevant to other bioorthogonal reactions are readily tolerated in both the reagent synthesis and methionine labelling, smoothly affording sulfonium-conjugates **4b,c** with 80-95% conversion to product. Biochemical reporter groups, such as myristyl- and fluorescein-derived esters **4d** and **4e**, are also readily transferred to exenatide. Interestingly, we found that sulfonium-conjugates of exenatide (such as **4f**) underwent reaction with the tertiary phosphine TCEP, a standard biochemical reagent, resulting in the cleavage of the labelling group and return of the parent exenatide **2b** in >90% conversion (Figure 3c)²⁵; the cleavage reaction also works for conjugates **5**, **6**, and **8** in comparable conversions and provides a stimulus-responsive means of reversing the bioconjugation reaction.

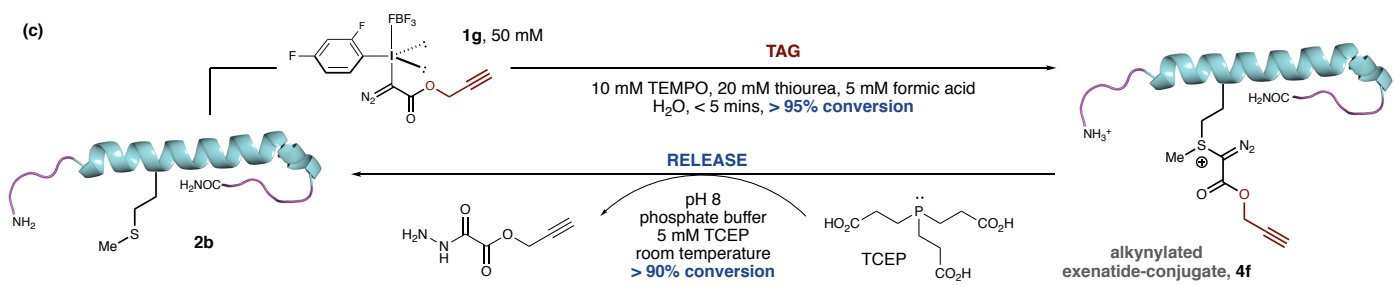
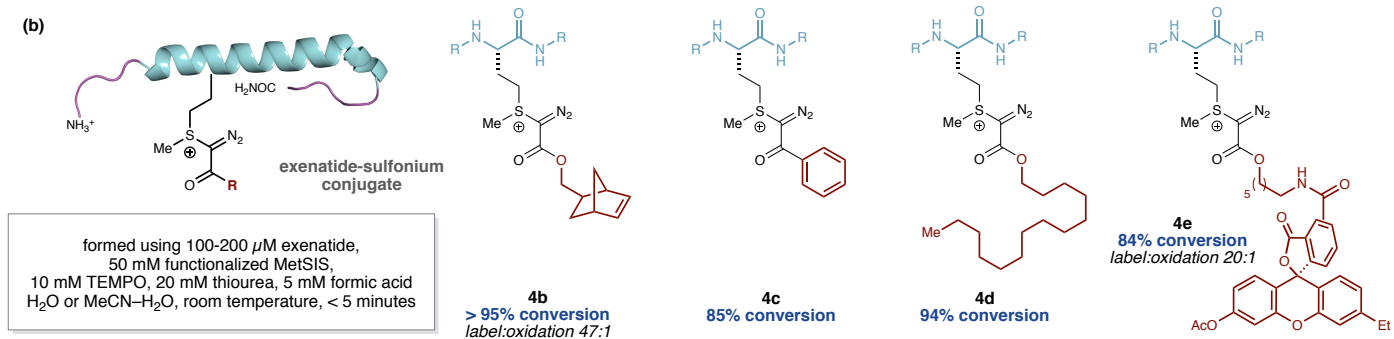
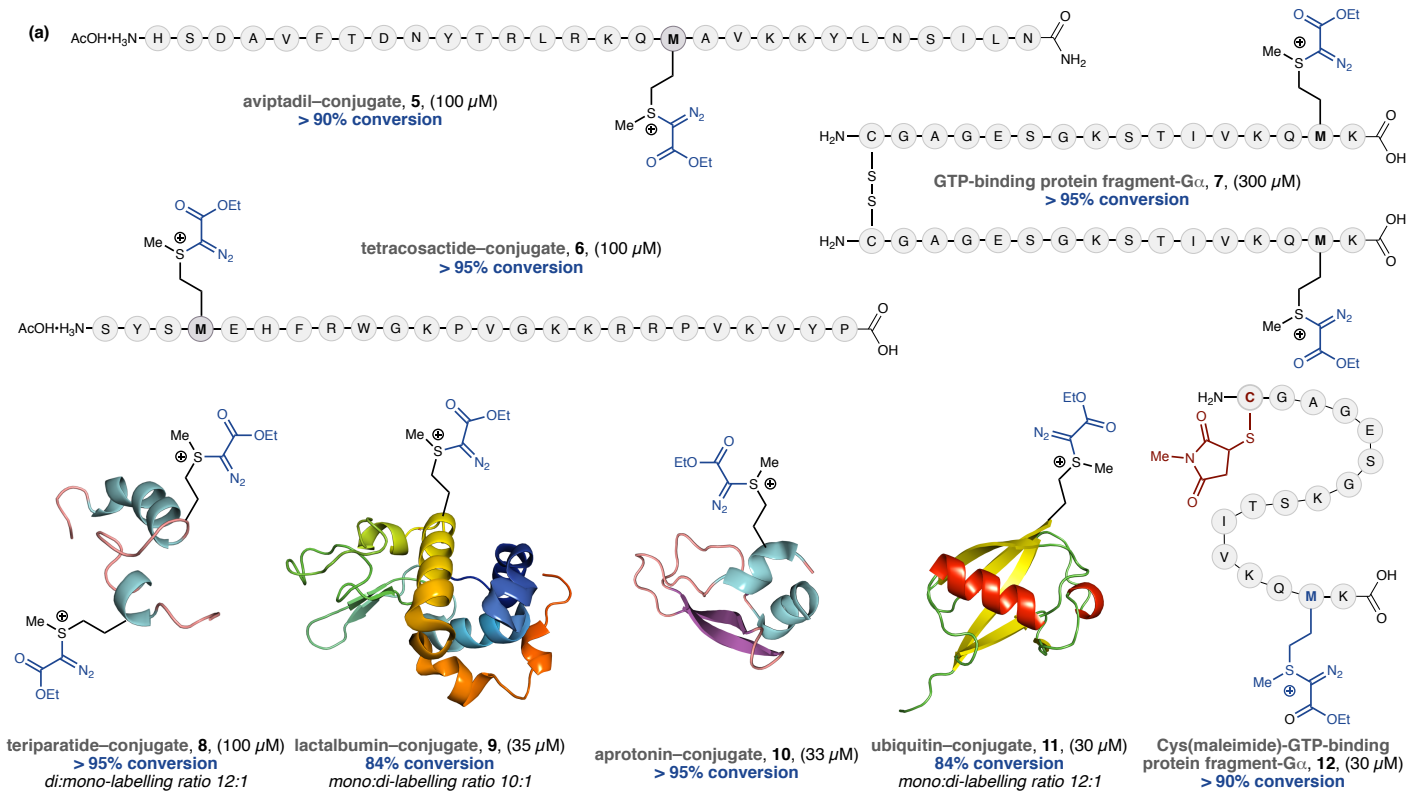
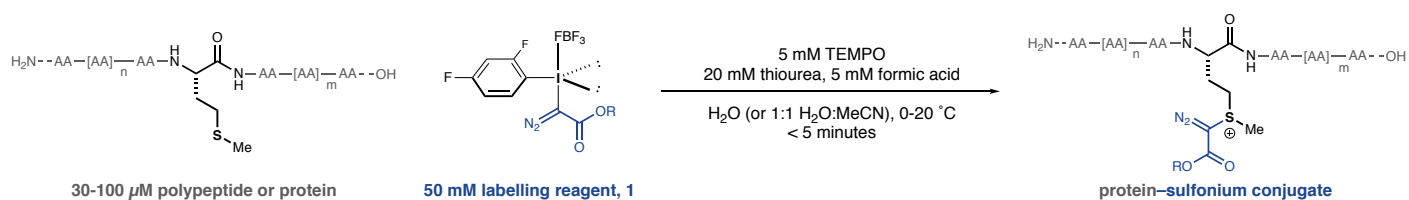


Figure 3. Scope of the methionine-selective bioconjugation strategy. (a) Range of polypeptide/protein substrates. (b) Functionalized iodonium reagents compatible with the bioconjugation. (c) A stimuli responsive strategy for reversing methionine bioconjugation.

Next, we turned our attention to exploring the multifaceted reactivity that we anticipated would be intrinsic to the high-energy methionine-derived conjugate. The electrophilicity of the diazo sulfonium-conjugate **4** prompted us to investigate the single electron transfer (SET) chemistry of this reactive motif enabled by visible light photocatalysis^{26,27}. Addition of a single electron to the diazo sulfonium conjugate **4** could result in intermediate **15**, which upon cleavage of the C–N₂ bond would form a putative radical ylide **15'** (Figure 4a). We envisaged two pathways through which we could exploit the reactivity of the previously unexplored radical ylide intermediate. Firstly, combining the radical ylide with photo-activated Hantzsch ester **13*** (from **13**) may lead to a reduction process resulting in the generation of a trialkylsulfonium motif, which would impart enhanced chemical stability to the protein-conjugate. Furthermore, deployment of a C-4 benzylated Hantzsch ester derivative (**14**, an established precursor for a benzyl radical)²⁸ to intercept the radical ylide species would lead to a C–benzylation product that could be used to introduce functional diversity to the protein-conjugate. We screened a range of photocatalysts under visible light conditions. When **3c** was irradiated with a 30 W lamp in the presence of *fac*-Ir(ppy)₃ and the Hantzsch ester **13**^{29,30}, we observed the formation of the reduced trialkylsulfonium product **17a** in 95% yield (determined by ¹H NMR, Figure 4b)³⁵. Using these conditions, we showed that a range of sulfonium-protein conjugates, including exenatide, glucagon and thioredoxin derivatives are reduced, with excellent conversions, to stable trialkylsulfonium species (**17b-e**, see the supplementary information S119 for details). Interestingly, reduction of a thioredoxin-derivative³¹ to its trialkylsulfonium-protein congener **17e** proceeds in high conversion without affecting its labile disulfide linkage, serving to highlight the mild nature of this protocol. Additionally, the methionine bioconjugation and photoreduction steps can be carried out in a one pot operation, significantly simplifying the overall process without compromising the yield or purity of the trialkylsulfonium product.

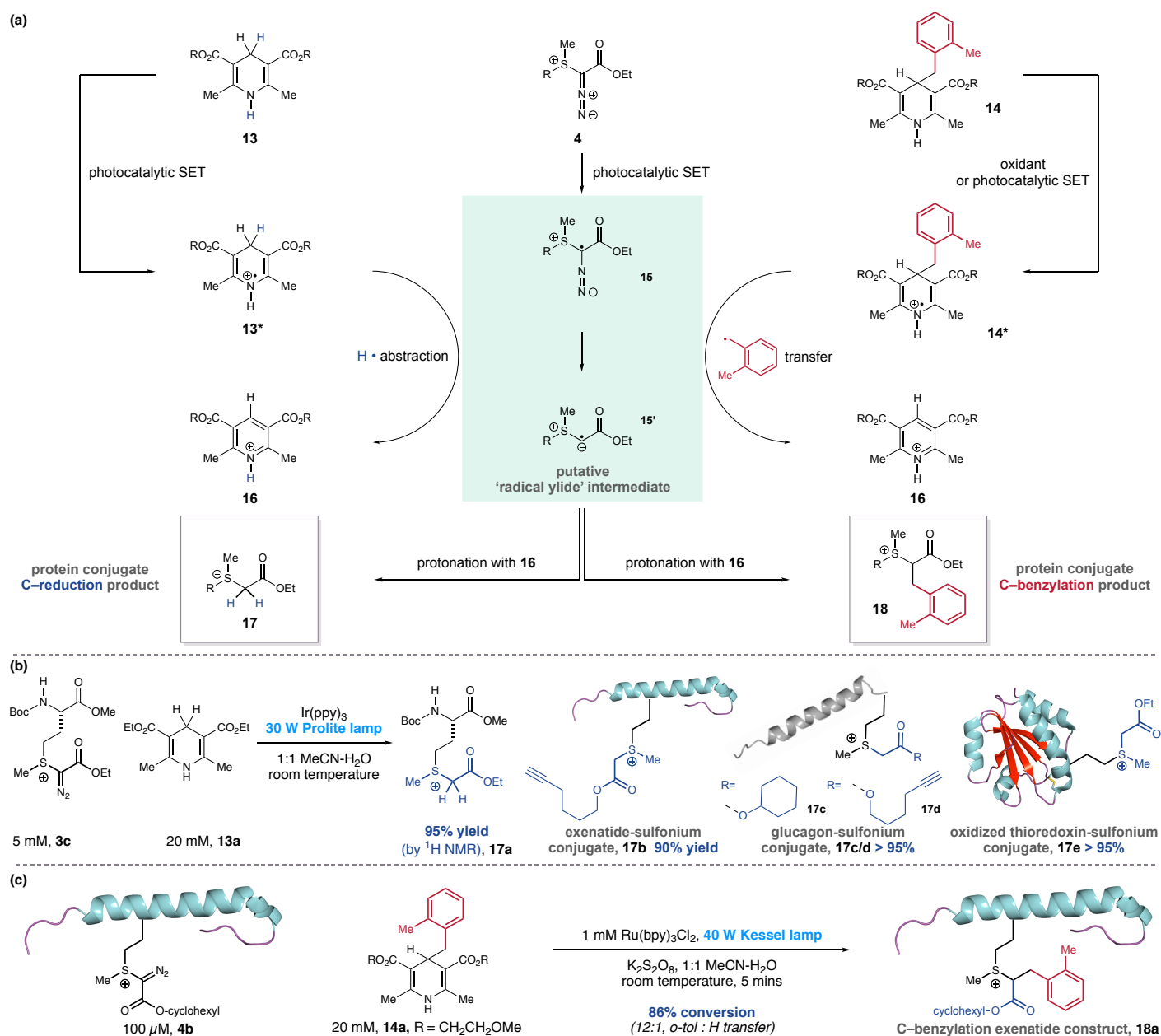


Figure 4. Exploiting the multi-faceted reactivity of the protein-sulfonium conjugate. **(a)** A photocatalytic design plan for secondary protein diversification. **(b)** A system for photo-redox mediated reduction of the sulfonium conjugate and examples of the substrate scope; in the case of **17e**, further reduction of C–S bond in **13** was not observed³⁵. Interestingly, the reduction of **13** proceeds in light, without the Ir-photocatalyst, but the yield was greatly diminished. **(c)** Secondary protein functionalization via photoredox radical cross coupling between diazo sulfonium-protein conjugate and C-4 substituted Hantzsch ester.

In testing the viability of the proposed C–benzylation using a modified Hantzsch ester derivative²⁸, we were delighted to find that treatment of the exenatide-sulfonium conjugate **4b** with o-tolyl Hantzsch ester derivative **14a**, under slightly modified photocatalytic conditions, led to cross-coupling and the formation of the C–ligation product **18a** in high conversion. This unique bioorthogonal protein functionalization reaction not only represents the first example of a synthetic radical-radical cross-coupling using a protein scaffold, but also provides a platform for bioorthogonal protein diversification wherein two distinct functionalities could be introduced sequentially at the same amino acid residue.

In summary, through the merger of methionine-selective bioconjugation and a new visible-light mediated photocatalytic reaction platform, information-rich synthetic constructs can be rapidly assembled by a two-step protocol directly from native proteins. The remarkable reactivity inherent to the methionine conjugate distinguishes the bioconjugation process from other methods. Moreover, the capacity for functional diversification, by tailoring the hypervalent iodine reagent and modified Hantzsch ester derivative, means that highly functional protein conjugates could be made readily available directly from native proteins.

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Supplementary Information

Included is a Materials and Methods section containing all experimental details and relevant compound characterization. Figs. S1 to S83, Tables S1 to S8, References, NMR Spectra

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Author contributions

MJG, MTT, JEN and MS conceived the project and designed the experiments. MJG, MTT, JEN and MS performed and analyzed the experiments. MJG, MTT and JEN wrote the paper.

Conflict of interest

The authors declare no conflict of interest

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