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Chemical Bioconjugation of Proteins in an Undergraduate Lab: One-pot Oxidation and Derivatization of the *N*-Terminus

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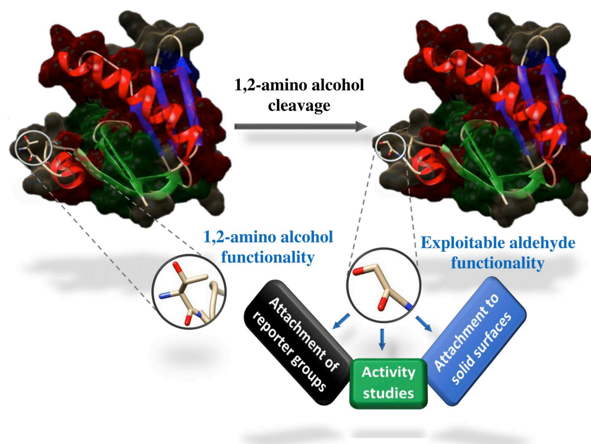
ABSTRACT

A laboratory experiment introducing the concept of chemical bioconjugation of proteins to undergraduate students in a therapeutically relevant context was developed. Initially students installed an aldehyde functionality into a protein *via* the oxidation of the *N*-terminal threonine residue of the 'cholera toxin subunit B' (CTB) protein, followed by subsequent modification *via* hydrazone addition under mild conditions with a chromophore bearing a distinct UV-vis absorption peak. Students determined the yield of the reaction to be *ca.* 11% by HPLC coupled to UV/vis spectroscopy, and developed key skills such as the preparation of stock solutions, and the chemical manipulation of proteins and analysis *via* HPLC. The experiment reported can be readily adapted for use with other proteins and may contribute to enhancing constructive alignment in interdisciplinary degree programmes at the chemistry/biology interface.

ABSTRACT GRAPHIC



KEYWORDS

Second-Year Undergraduate, Biochemistry, Organic Chemistry, Aldehydes/Ketones, Bioorganic Chemistry, Proteins/Peptides.

INTRODUCTION

Undergraduates enrolled in interdisciplinary programmes such as pharmacy, biochemistry and natural sciences, commonly undertake discrete "biology" and "chemistry" practical experiments, but too rarely actively experience the integration of these fields. This is despite a well established appreciation of the potential benefits of undergraduate training through integrated practical laboratory experiences[1,2]

particularly in preparing students for careers as research scientists where they are likely to be confronted with "real-world observations that do not separate well into conventional disciplines".[3] An undergraduate experiment was therefore designed focussed in the pharmaceutically relevant field of protein bioconjugation to allow students to experience the increasingly interdisciplinary nature of chemical biology in industry, applying principles from synthetic organic chemistry, biochemistry, analytical chemistry and separations science. Student learning goals for the experiment include: 1) developing accurate weighing and measuring skills required for protein modification; 2) being able to synthesize a protein-small molecule conjugate and analyze using high performance liquid chromatography; 3) using a calibration curve to calculate protein concentration from the peak area of a chromatogram; 4) applying small molecule organic chemistry knowledge to rationalize the results of a protein bioconjugation, and the possible byproducts observed; and 5) rationalizing as to whether protein concentration determined in the experiment are an overestimate or underestimate.

While undergraduate experiments focused solely on protein purification and characterisation have already been described,[4–7] due to the expensive instrumentation commonly associated with purification and analysis,[4] few examples describing the chemical bioconjugation of proteins exist. It was anticipated that such an experiment could be employed in conjunction with other chemical biology practical experiments in the future, such as the "Just Click It" introduction to click-chemistry by Sharpless and co-workers,[8] to give undergraduates a rounded introduction to the field.

Although many post-translational enzymatic modification of proteins are performed by organisms in nature,[9–11] at present the ability to chemically modify proteins in practice within the constraints of an undergraduate laboratory experiment is limited. Firstly, the synthetic procedures utilised for targeting protein scaffolds need to be biologically compatible (e.g. aqueous conditions, mild temperature, pH 6-8), so as not to denature the protein[9,10] and additionally to be able to selectively target a single site within a protein, therefore reducing complexity of the characterisation to be performed by the student. Notably, a general method to achieve this level of chemoselectivity and regioselectivity is still a goal under study in many research labs.[9] However chemical bioconjugation of proteins can be highly chemoselective if a unique functionality can be targeted, such as the *N*-terminus. *N*-terminal threonine and serine residues contain 1,2-amino alcohol functionalities.[12,13] The mild periodate oxidation of these moieties introduces a reactive glyoxylamide functional group **1** (Figure 1) only at the protein *N*-terminus.[12–14] The reactivity of the glyoxylamide aldehyde functionality can then be exploited to decorate macromolecular proteins with small molecule substrates including drug molecules, affinity tags, reporter groups (spectroscopically active moieties) or even solid surfaces by grafting. Indeed, biomolecular hybrids in this mold have a broad range of applications such as biocatalysis, the study of protein function, and the development of powerful new biomedicines and biosensors.[15–18]

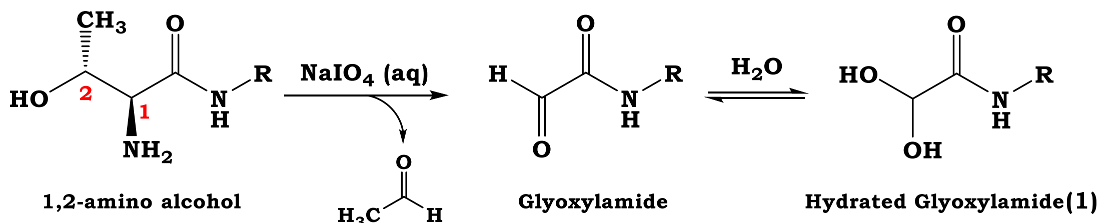


Figure 1. The periodate oxidation of the 1,2-amino alcohol functionality of an *N*-terminal threonine residue to a glyoxylamide **1** functional group.

The cholera toxin (CT), secreted by *Vibrio cholerae* and estimated to cause up to 142,000 deaths per annum worldwide,[19] is an example of a protein complex bearing an *N*-terminal residue which has previously been exploited in chemical modification strategies.[20] The CT complex is comprised of two types of subunits: non-toxic cholera toxin subunit B (CTB), which self-assembles to form a pentameric ring, and toxic cholera toxin subunit A (CTA), which threads through the pore at the center of the CTB pentamer and associates to form the complete CT complex (Figure 2).[16,21] CTB pentamers participate in multivalent binding to GM1 glycolipids present on the surface of intestinal epithelial cells, an interaction which facilitates entry of CT into the cell by receptor-mediated endocytosis.[16,21] Once inside the cell, the CTA monomer is released from the CTB pentamer and subsequently perturbs cellular regulation of chloride ion movement, leading to severe diarrhoea and vomiting.[21]

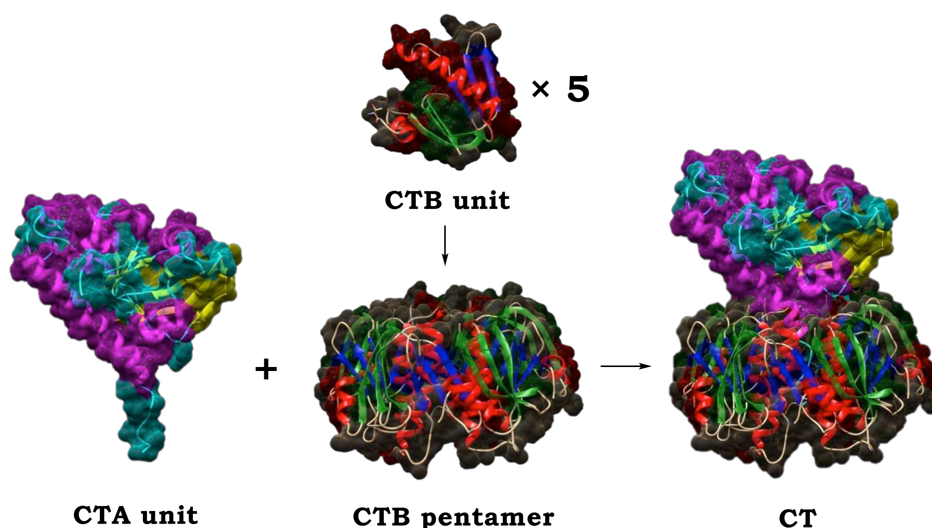


Figure 2. The structure of cholera toxin (CT).

Recently, the utility of periodate oxidation of *N*-terminal threonine residues to glyoxylamides on a mutant CTB pentamer was showcased in the construction of a pentavalent neoglycoprotein. When derivatized through oxime bonds with aminooxy-bearing GM1 ligands, the *N*-terminally modified CTB mutant acted as the most potent inhibitor of the cholera toxin reported in the literature to date.[16] As many pathogens and protein toxins adhere to their target cells by multivalent binding to specific cell-surface molecules, general inhibition strategies using protein-based scaffolds as multivalent binders are therefore of increasing medicinal interest.[22] Inspired by this precedent and application in a biomedical context, and the fact that CTB is readily available from commercial suppliers, the pentameric protein scaffold was chosen for developing an undergraduate laboratory bioconjugation experiment. Herein we describe a laboratory experiment where students perform the facile oxidation of the *N*-terminal threonine residue of CTB using sodium metaperiodate, followed by the aniline-catalyzed hydrazone condensation of glyoxyl-CTB with 3-methyl-2-benzothiazolinone hydrazone (MBTH) and subsequently characterise this adduct using HPLC coupled to UV-visible absorption spectroscopy (Figure 3). The experiment showcases state-of-the-art methodology for small molecule-protein bioconjugation in a therapeutically relevant

context, while enabling students to develop practical skills and experience in the field of chemical biology, including introductory protein handling and manipulation, preparation of stock and buffer solutions, and protein/small molecule analysis *via* UV/vis spectroscopy and HPLC.

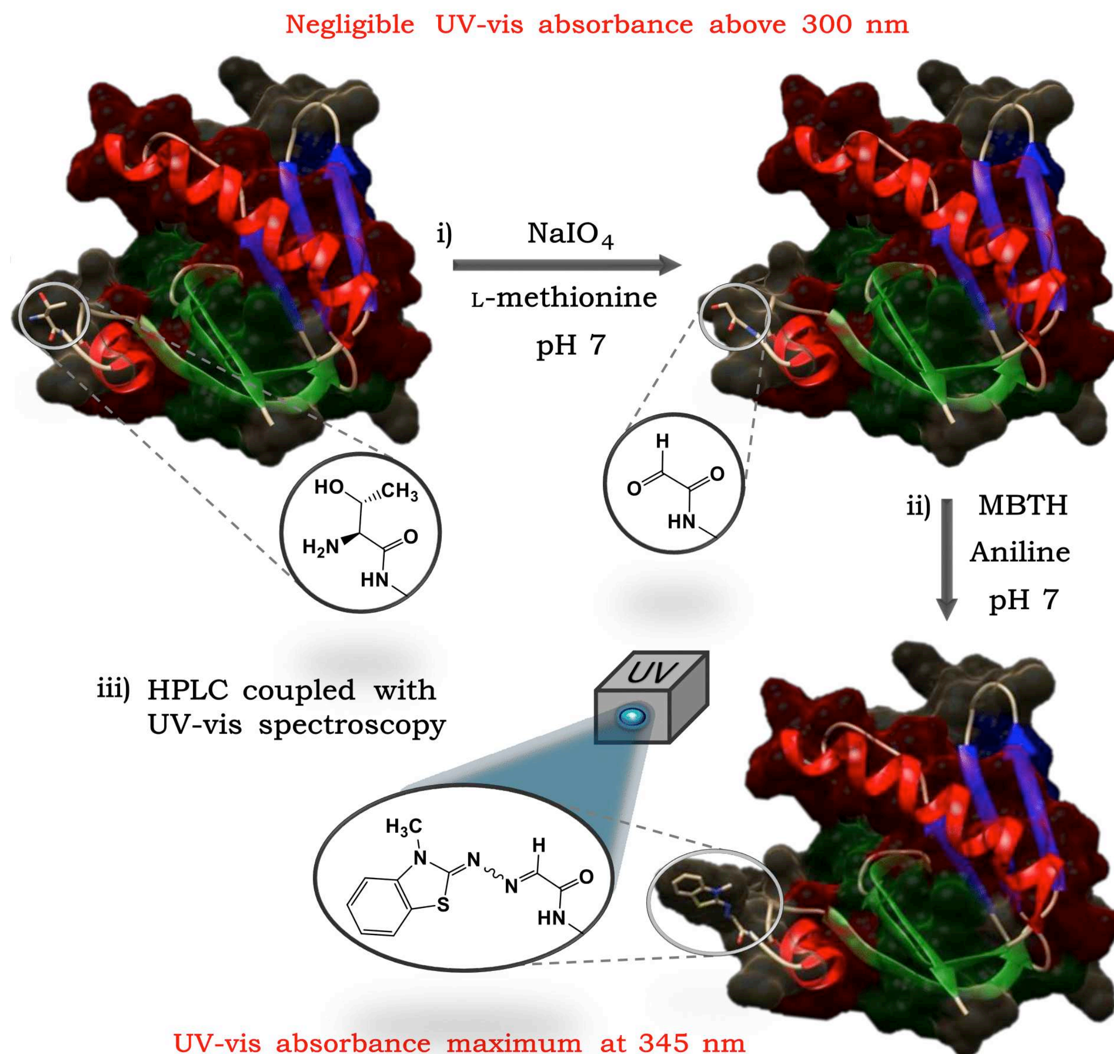


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Figure 3. The preparation and detection of a CTB-MBTH adduct. The CTB-MBTH adduct can be distinguished from 'untagged' proteins using a UV-vis spectroscopy band with a maximum at 345 nm. Wavy bond line indicates stereochmistry is unknown.

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EXPERIMENTAL METHODS

N-terminal Oxidation of CTB

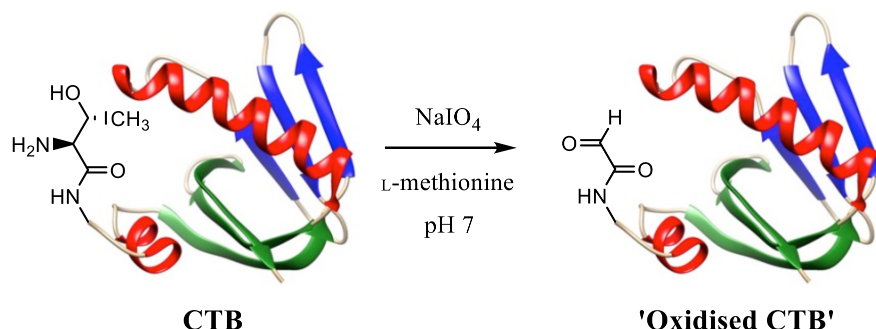

Figure 4. The oxidation of the *N*-terminal threonine residue of CTB using sodium metaperiodate to yield an exploitable aldehyde functionality.

Stock solutions of phosphate buffer (10 mM, pH 7), CTB protein (0.46 mM), L-methionine (60 mM), $NaIO_4$ (32 mM), MBTH (21 mM), and aniline (200 mM) were prepared (see Supporting Information p S3)

Students added a 25 µL aliquot of 10 mM pH 7 phosphate buffer solution to a 0.5 mL Eppendorf tube. After this addition, a 21 µL aliquot of the CTB stock solution (delivering 10 nmol CTB) was added to the Eppendorf tube, followed by 1.7 µL of $NaIO_4$ stock and 1.7 µL of L-methionine stock, yielding *ca.* 50 µL of reaction solution. The Eppendorf tube was then sealed and inverted to facilitate mixing of the reaction solution. The Eppendorf tube was placed in a dark place and the reaction allowed to proceed for 15 min at room temperature, yielding oxidized CTB (Figure 4). Note that the volumes of phosphate buffer and CTB stock solution added to the Eppendorf tubes can be varied depending on the concentration of the CTB stock available.

Formation of a CTB-MBTH adduct

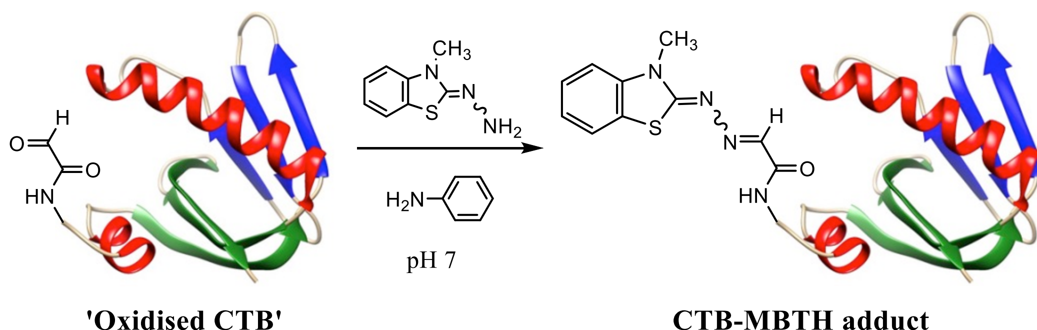

Figure 5. The formation of azine-derivatized CTB *via* the reaction of MBTH with the aldehyde functionality introduced at the *N*-terminus of CTB.

Students then added 50 µL of MBTH hydrochloride and aniline stock solution to the Eppendorf tube containing the oxidized CTB. The Eppendorf tube was subsequently sealed and inverted, ensuring the mixing of the reaction solution, and placed in a dark place. The reaction was then allowed to proceed for 20 min, yielding the CTB-MBTH adduct (Figure 5).

Characterization via HPLC

155 A C-18 column (4.6 x 150 mm, 5μM) was used with an isocratic mobile phase (70% acetonitrile (with 0.1% formic acid) and 30% deionized water (with 0.1% formic acid) at a flow rate of 700 μL min^{-1}, allowing a sample to be run by a student in approximately 6 min. The mobile phase was sonicated before use (until gas evolution ceased) to prevent bubbles entering the column. The UV unit of the HPLC instrument stack was set to detect absorbance at 345 nm. The injection loop of the HPLC instrument was rinsed
160 with 100 μL of distilled water between sample loadings. Students collected chromatogram traces for their products when loading 40 μL samples of their azine-derivate solution into the injection loop, with each sample taking around 5 min to run.

A calibration curve was constructed by loading 40 μL of serine-MBTH azine-derivative solutions of known concentrations (see Supporting Information p S7) onto the
165 column. It is possible to use the equation fitted to this curve to calculate the concentration of the CTB-MBTH adduct from the peak area of the chromatogram peak.

HAZARDS

L-Methionine, sodium phosphate monobasic dihydrate and sodium phosphate dibasic
170 dodecahydrate are not considered harmful substances, although normal lab practice should be followed. Cholera Toxin subunit B, and the CTB-MBTH adduct are handled in phosphate buffer solution which may be harmful by inhalation, ingestion or skin absorption, and may cause eye, skin or respiratory system irritation.

Sodium metaperiodate is a strong oxidizing agent and may cause fire or explosions.
175 It can also cause severe skin burns, eye damage, damage the thymus gland through prolonged or repeated exposure, and is toxic to aquatic life. As such, sodium metaperiodate should be kept away from heat, hot surfaces and ignition sources.

Aniline is a suspected carcinogen, and toxic if swallowed, in contact with skin or if inhaled, as is 3-methyl-2-benzothiazolinone hydrazone hydrochloride hydrate.
180

RESULTS

The experimental procedure detailed was completed by students as part of a summer school within an undergraduate chemistry degree program, where students had little or no prior experience of integrated chemical biology experiments, and completed the
185 experiment within 5 h.

In the first stage of the experiment, students prepared stock solutions and oxidized the *N*-terminal threonine of CTB using sodium metaperiodate (with the average yield of this oxidation step repeatedly pre-determined by ESI-MS to be 90%, see Supporting Information p S4 for mass spectrometry characterisation). Oxidations were conducted
190 at pH 7 and at room temperature for 15 min, which enabled the introduction of an aldehyde functionality with no observed protein degradation.[12] Although periodate oxidation of *N*-terminal threonine or serine residues proceeds rapidly; given sufficient time, periodate will also oxidize any amino acid.[12,13,23] Therefore to minimize the occurrence of such side reactions (which could denature the protein), the free amino
195 acid L-methionine was added to the reaction solution.

The newly introduced *N*-terminal protein aldehyde was reacted efficiently with MBTH in the presence of aniline within 20 min to afford the CTB-MBTH azine conjugate without the need for intermediate protein purification. Students performed characterization and determined the yield of the CTB-MBTH adduct formation using
200 HPLC-coupled UV-vis spectroscopy. Students readily identified the CTB-MBTH azine

adduct as a sharp peak on the chromatogram trace of the reaction solution (Figure 6). The stack-plot of student chromatograms in Figure 6 demonstrates the robust and reproducible elution of the CTB-MBTH adduct at approximately 4 min, and that all students were able to successfully perform the bioconjugation reaction, providing
205 qualitative evidence that they had developed accurate weighing and measuring skills during the experiment, highlighted as a learning objective. Furthermore, students were able to use the area of this peak in conjunction with a calibration curve (constructed using known concentrations of a serine-MBTH adduct, Supporting Information Figure S6, p S7), to accurately determine their yield of the CTB-MBTH adduct. The average
210 concentration of the CTB-MBTH adduct achieved and calculated by students was determined to be *ca.* 11 μM, which equates to an 11% yield for the formation of the CTB-MBTH adduct (assuming the yield of oxidized CTB to be 90%). On the basis of responses to questions posed at the end of the experiment, 60% of students were also able to apply their knowledge of small molecule organic chemistry to successfully
215 propose an MBTH adduct that would be formed as a by-product in the reaction, and 90% were able to rationalise and justify whether their calculated yield was an over or underestimate of the reaction conversion.

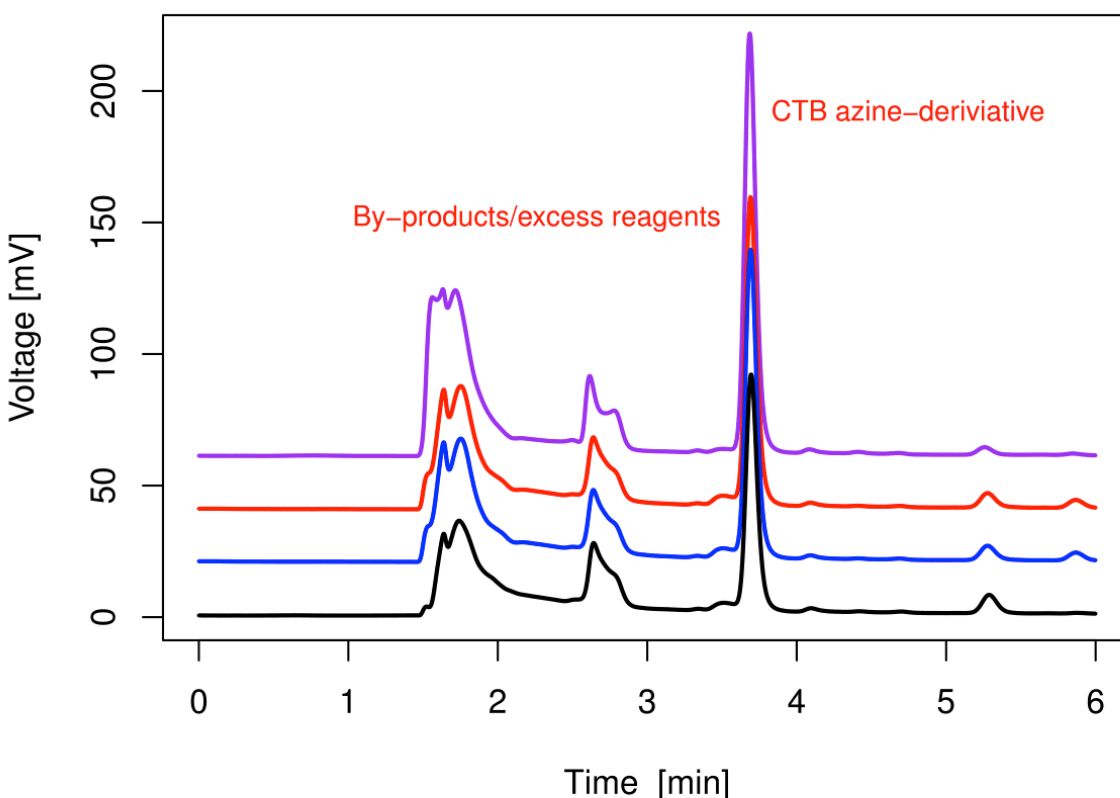

Figure 6. HPLC-coupled UV-visible spectroscopy chromatogram stack-plot of student acquired data, showing a consistent
220 peak for the CTB-MBTH azine derivative.

Students found some practical aspects of the experiment challenging, specifically the handling and accurate pipetting of small reagent volumes, however inaccuracies could

225 be reduced by encouraging students to repetitively practice pipetting and weighing specific volumes of water.

Throughout this experiment the students were exposed to key skills intrinsic to state-of-the-art chemical biology studies which they are likely to encounter in both research or industrial context in the future, including the preparation of stock
230 solutions, the handling and chemical manipulation of proteins, and the analysis of bioconjugation reactions using spectroscopy. Such an experiment required them to apply theoretical principles from the fields of synthetic organic chemistry, biological chemistry, analytical chemistry and separations science, thus serving to reinforce their theoretical understanding of the siloed fields, but also visualise connections across the
235 interfaces of chemistry and biology.

CONCLUSIONS

Through the use of the techniques described, a protein bearing a surface-exposed *N*-terminal serine or threonine residue can be "tagged" and characterized in a low-cost
240 and efficient manner. Such an experiment allows students to undertake cutting-edge chemical biology through mimicking a working example of bioconjugation for applications as a therapeutic agent within the constraints of an undergraduate laboratory class. As such, this laboratory experiment is likely to contribute towards enhancing the degree of constructive alignment in interdisciplinary programmes such
245 as pharmacy, biochemistry and natural sciences.

ASSOCIATED CONTENT

Supporting Information

A student lab manuscript, graduate teaching assistant manuscript, and a Supporting
250 Information pdf file detailing considerations made during experimental design, a methods section for technicians, CTB protein production and mass spectrometry, and calibration curve construction are available via the internet at http://pubs.acs.org.

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