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Isotope-Coded Maleimide Affinity Tags for Proteomics Applications

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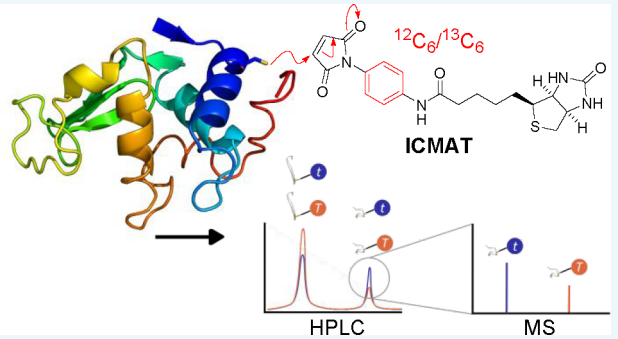
ABSTRACT: Isotope-coded affinity tags (ICATs) are valuable tools for mass spectrometry-based quantitative proteomics, in particular, for comparison of protein (cysteine-residue) thiol oxidation state in normal, stressed, and diseased tissue. However, the iodoacetamido electrophile used in most commercial ICATs suffers from poor thiol-selectivity and modest rates of adduct formation, which can lead to spurious results. Hence, we designed and synthesized three ICATs containing thiol-selective *N*-alkylmaleimide electrophiles (isotope-coded maleimide affinity tags = ICMATs) and assessed these as mass spectrometry probes for ratiometric analysis of lysozyme and muscle proteomes. Two ICMAT pairs containing butylene/D_8-butylene linkers were effective MS probes, but not ideal for typical proteomics workflows, because peptides bearing these tags frequently did not coelute with HPLC. A switch to a phenylene/$^{13}C_6$-phenylene linker solved this issue without compromising the efficiency of adduct formation.



INTRODUCTION

Chemical probes for covalent protein labeling have proven invaluable in quantitative proteomics. Isotope-coded affinity tags (ICATs) were among the first probes used for high-throughput quantitation workflows. While other techniques and labels are now more commonly used for general quantitative proteomics applications, probes that alkylate cysteine residues (targets of cellular oxidation events) are valuable for quantitative redox proteomics.[1−6] Typical ICATs comprise three components: a reactive electrophile that alkylates cysteine thiols, a biotin residue that allows enrichment of tagged peptides, and an isotopically variable linker for ratiometric analysis by mass spectrometry (MS).[2] Additional functionality can be introduced into the ICAT, such as a ^{14}C or fluorescent visualization motif[7] and/or a cleavable linker that simplifies, and enhances the sensitivity of, MS analysis.[7−9]

A schematic illustrating the principle of ICAT methodology is set out in Figure 1. Protein extracts from tissue samples or cell culture are denatured and treated with the light tag (t). Ideally, only polypeptides with free thiol groups are covalently labeled in this first tagging step. The entire sample is then reduced, converting oxidized cysteine residues to the free thiols, which are then labeled with the heavy tag (T). The mixture is then trypsinized, and tagged peptides are separated from untagged peptides using (strept)avidin affinity chromatography or pull down. Finally, LC−MS/MS analysis allows protein identification, and ratiometric comparison of light:-heavy tagged peptides reports on the ratios of the reduced:oxidized cysteine residues present in the original mixture. ICAT methodology is generally faster, more convenient, higher throughput, and more accurate than gel-based proteomics workflows.[10,11]

The heavy ICAT typically incorporates a polydeuterated alkyl chain or ^{13}C-enriched linker, while the corresponding light ICAT contains the naturally abundant isotopes. A mass difference of at least 5 Da is preferred, to enable tagged-peptide ion resolution, which may otherwise be compromised by naturally abundant ^{13}C.[10] In principle, the light/heavy ICAT pair are chemically identical, meaning the reactivity is unaffected on changing from a light probe to a heavy probe.

An iodoacetamide (IAM) moiety is most commonly used as the thiol-reactive component in ICATs. Although this electrophile was originally chosen to be thiol-specific,[1] it has since been found that that N-alkylation (e.g., of histidine residues) is not just common, but it is the dominant conjugation mode for IAM using typical protocols.[12] Indeed, many undesired side-products have been observed in the attempted labeling of cysteine thiol groups using IAM, including alkylation of N- and C-terminal amino and carboxylate groups, respectively.[13] IAM also doubly alkylates the ϵ-amino group of lysine residues, resulting in a tertiary amine (2-acetamidoacetamide) adduct, which has been

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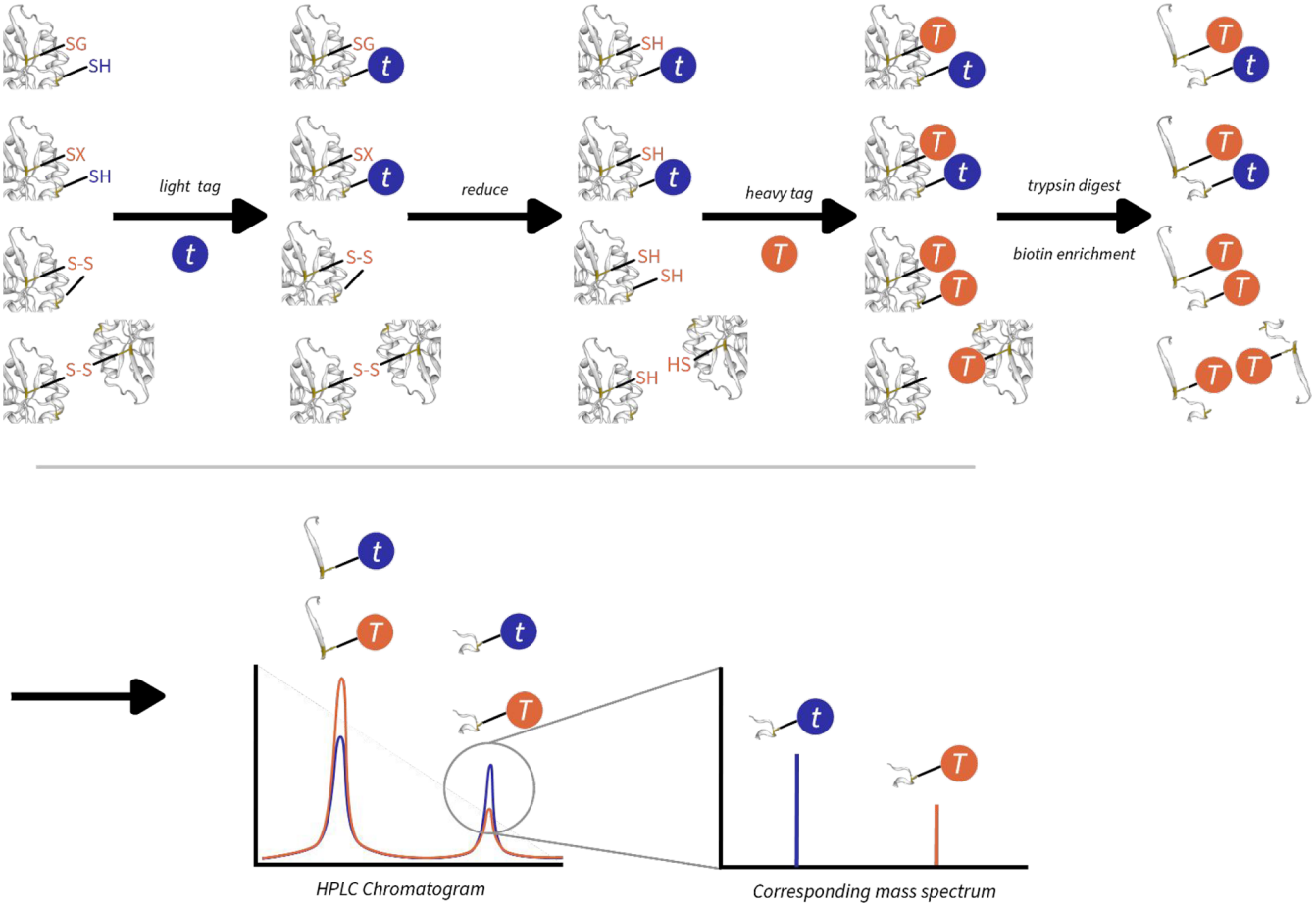

Figure 1. Schematic example of ICAT methodology for the interrogation of protein−cysteine oxidation state (OxICAT). G = glutathione; SX = an undefined oxidized cysteine sulfur; T = heavy tag, t = light tag.

misidentified as the diglycine modification characteristic of protein ubiquitination, post-trypsin digestion.[14]

Alkylation of thiols by soft Michael acceptor electrophiles is considerably faster and more selective than with IAMs; *N*-alkylmaleimides (NAMs) have been most widely studied. The reaction of 2-nitro-5-thiobenzoic acid with *N*-ethylmaleimide (NEM) was 20-fold faster than with IAM.[15] In an analysis of murine skeletal muscle tissue, 4 h of incubation with IAM was necessary to alkylate all free thiols, but was complete in just 4 min with NEM.[16] Indeed, alkylation of protein thiols by IAM may be incomplete even after 6 h of incubation.[17] For practical purposes, the relatively slow reaction kinetics means that large stoichiometric excesses of IAMs are required to complete timely global thiol labeling. Much smaller excesses are required for NAMs. In isolated myofibril preparations, complete alkylation of thiols required a 1000-fold excess of IAM, but only a 125-fold excess of NEM.[16] The larger excess required for IAMs exacerbates their first major drawback, poor chemoselectivity, leading to increased mislabeling of nucleophilic functional groups other than thiols, and consequent misidentification of peptides. In addition, IAMs are photolabile, and must be handled in the dark.[2,18] Overall, these limitations of IAMs reduce the performance of the commercial ICATs, especially when the amount of protein is unknown, as using a molar excess can lead to indiscriminate "overlabeling".

d_5-NEM has been used alongside (light) NEM in conjunction with gel-electrophoresis for MS-ratiometric proteomics.[10] There are also several patents that claim the use of biotinylated ICATs incorporating a maleimide terminus;[19−23] however, to the best of our knowledge, no such chemical probes have been published in peer-reviewed journals.

We recently reported the optimization of sample preparation workflow using commercial biotin maleimide.[24] Herein, we describe the design, synthesis, and preliminary evaluation of bespoke proteomics probes incorporating a maleimide moiety, which we call ICMATs (isotope-coded maleimide affinity tags).

RESULTS AND DISCUSSION

The key features required in the new thiol-reactive probes for proteomic mass tagging are summarized in Figure 2. First, for the reasons described above, a terminal maleimide moiety was chosen as the thiol-selective trap. A biotin residue comprises the other terminus of the ICMAT, to facilitate purification/enrichment of tagged peptides, through (strept)avidin affinity

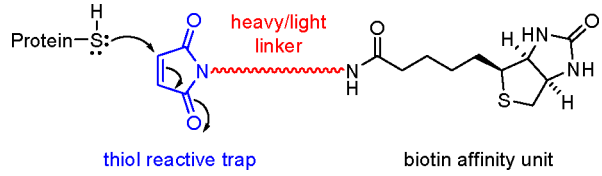

Figure 2. Generic structure and key features of ICMATs.

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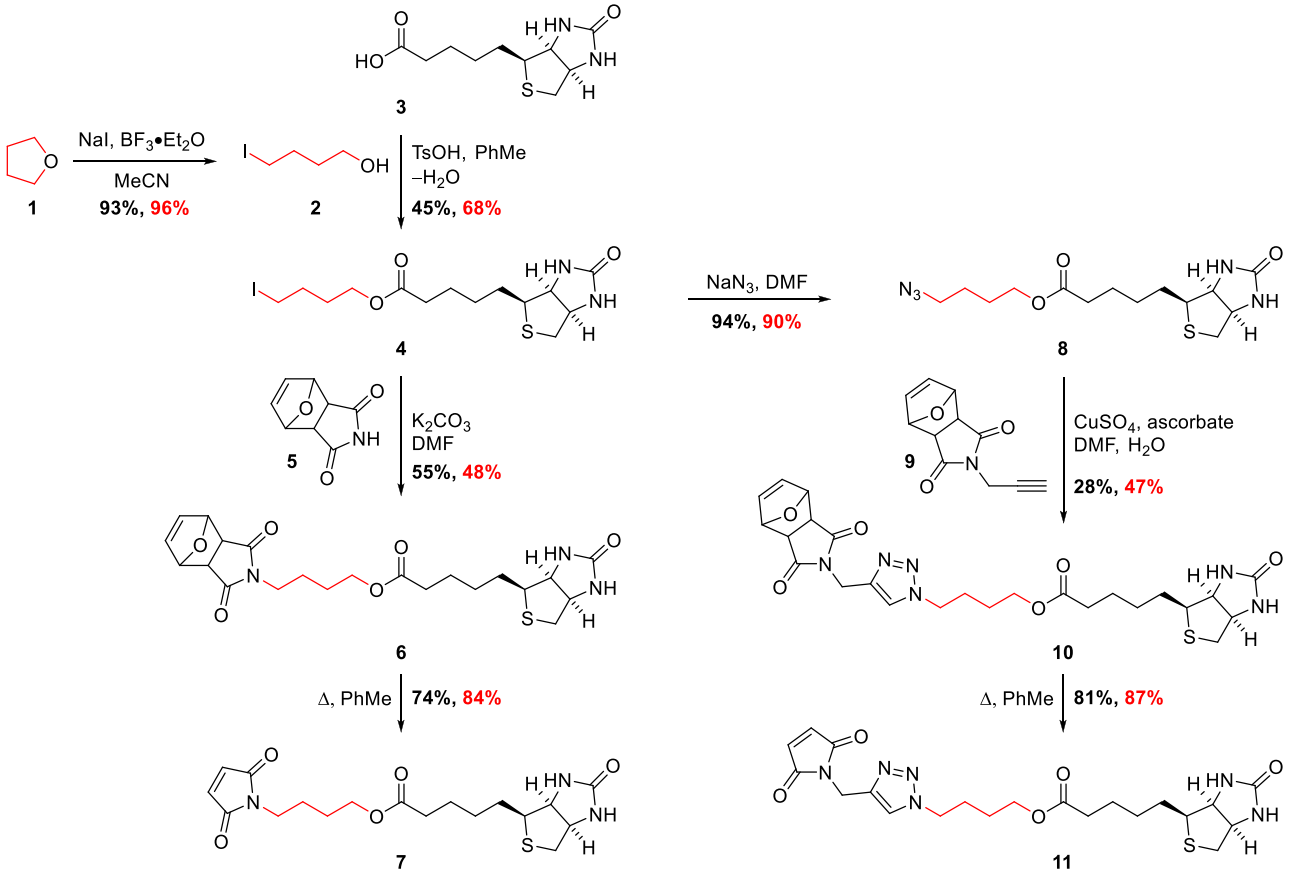
Scheme 1. Synthesis of Light and Heavy* (d_8) ICMAT-1 (7/7*) and ICMAT-2 (11/11*)[a]



[a]The red linker indicates the positions of perdeuteration. Yields for the light and heavy isotopologues are in black and red, respectively.

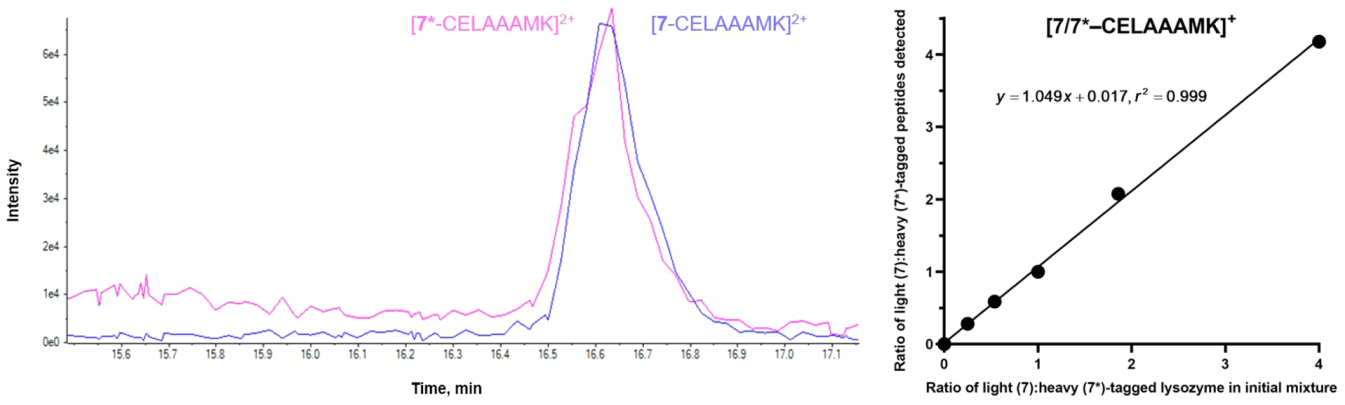


Figure 3. Left: Representative extracted-ion chromatogram for the ICMAT-1 (7/7*)-tagged peptide CELAAAMK derived from lysozyme. Right: Representative standard curve based on the same tagged peptide. Ratios of light:heavy-tagged peptides were determined by extracted-ion chromatogram peak integral.

chromatography/pull down. Finally, the linker connecting the two components above must be appropriate for isotopic labeling; that is, the heavy isotopologue must be synthetically accessible from a reasonably priced, commercial starting material. In addition, ideally the heavy and light linkers have a mass difference of at least 5 Da to achieve clear resolution in the mass spectra of peptide−probe adducts. These points were considered in the design of the ICMATs described below.

Synthesis of $^1H/^2H$-Labeled ICMATs. The syntheses of the initially targeted ICMAT-1 (7) and ICMAT-2 (11) are outlined in Scheme 1. All steps were first conducted with unlabeled (light) materials, followed by repetition with the d_8 (heavy) isotopologues (denoted by *). Tetrahydrofuran (THF, 1) is a convenient starting point, as d_8-THF is reasonably inexpensive and readily available due to its use as a solvent for NMR spectroscopy. Ring opening of THF (1)[25] provided 4-iodobutan-1-ol (2). Fischer esterification of D-biotin (3) with this alcohol gave 4, from which the syntheses diverged. Alkylation of the protected maleimide 5[26] with iodide 4 gave 6, which was subjected to a retro-Diels−Alder

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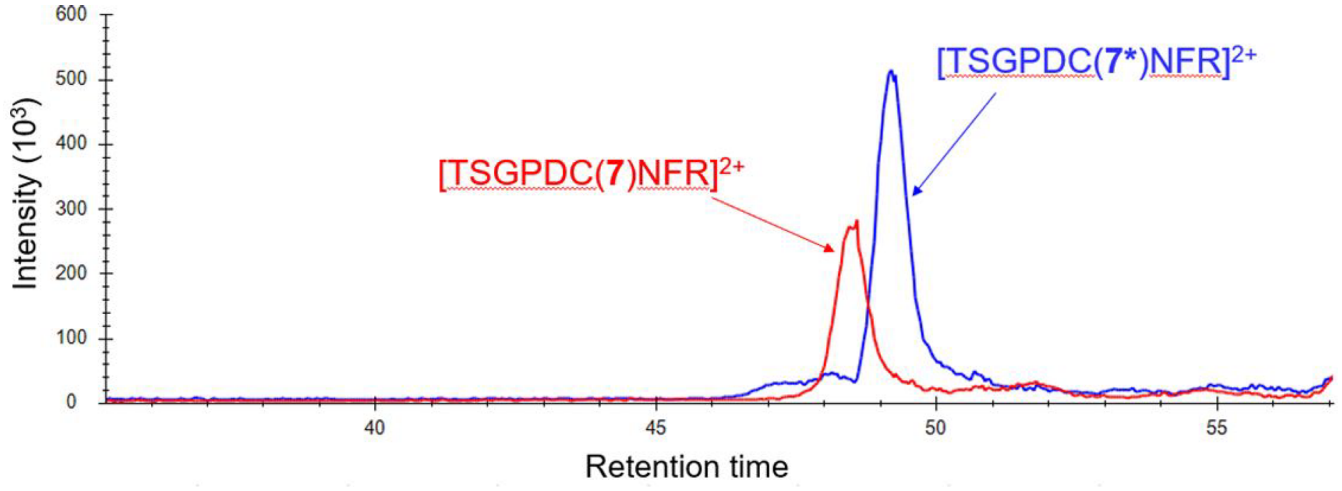


Figure 4. Extracted-ion chromatogram showing the different elution times of light- [TSGPDC(7)NFR]$^{2+}$ (m/z 696.2896, elution time 48.6 min) and heavy-tagged [TSGPDC(7*)NFR]$^{2+}$ (m/z 700.3147, elution time 49.2 min) peptide derived from mouse muscle titin.

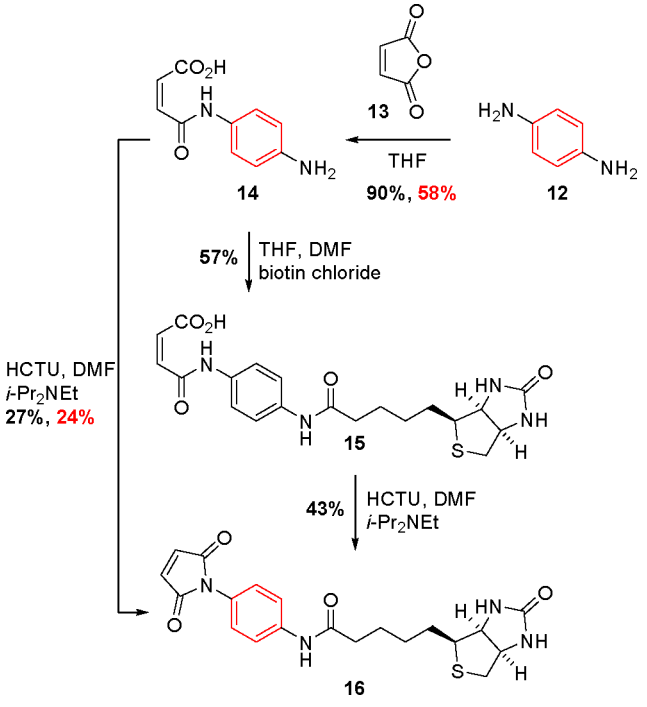
reaction to give ICMAT-1 (**7**) containing a simple butylene linker. Alternatively, conversion of iodide **4** to the corresponding azide **8**, followed by copper-catalyzed cycloaddition with terminal alkyne **9**, gave triazole **10**, which was thermally unmasked to provide ICMAT-2 (**11**).

Evaluation of ICMAT-1. The suitability of the ICMAT-1 pair 7/7* for ratiometric quantification of light- and heavy-tagged proteins was assessed using a model protein, lysozyme, which was chosen because it contains eight cysteine residues. Briefly, commercial chicken egg lysozyme was reduced to cleave all disulfide bonds, then exhaustively thiol-labeled by treatment with ~30 equiv of light **7** or heavy **7***. The light- and heavy-tagged lysozyme samples were mixed in various ratios and then subjected to a standard proteomic workflow for bottom-up MS analysis (trypsin digestion, peptide cleanup, LC–MS/MS).[27] A single tagged peptide was observed and, as expected, automated MS/MS analysis (see Experimental Procedures for details) confirmed this as being derived from lysozyme. Encouragingly, the light- and heavy-tagged peptides coeluted on HPLC (Figure 3, left). Relative quantification of the light/heavy-tagged peptides by comparison of peak integrals very closely matched the prepared ratios (Figure 3, right).

ICMAT-1 pair 7/7* was then used to interrogate the oxidation status of cysteine residues in the proteome of mouse muscle tissue, which was subjected to a typical OxICAT-type workflow.[28] Homogenized tissue was incubated with **7** (0.29 nmol per μg tissue). Tags were incorporated efficiently—no untagged peptides were detected, as reported by Mascot search engine from the MS/MS output files—and did not interfere with the identification of the major muscle proteins (e.g., actin, myosin, titin, tropomyosin, and troponin C). However, unlike in the model protein lysozyme, the light and heavy isotopologues in a subset of tagged peptides from muscle tissue had slightly different HPLC retention times (e.g., Figure 4). This effect, which is usually attributed to the slightly greater hydrophobicity of protiated molecules relative to their deteurated isotopologues,[29] complicates ratiometric analysis, and has been noted previously with ICATs.[7,8] Surprisingly, in this case, the deuterated tagged peptide has a longer retention time, indicating a stronger interaction with the C_{18} stationary phase. Thus, while ICMAT-1 (7/7*) and, by analogy, ICMAT-2 (**11**/**11***) are suitable for direct proteome analysis, for example, using MALDI–TOF/TOF, or for use in conjunction with gel-based fractionation, they are not ideal for the most powerful and common protocols that incorporate an HPLC fractionation step to improve resolution. Accordingly, we designed a $^{13}C/^{12}C$ ICMAT pair, in which the isotopologues were expected to have identical chromatographic mobilities.

Synthesis of $^{12}C/^{13}C$-Labeled ICMAT-3. $^{13}C_4$-THF is not commercially available, and in any case would only provide an M+4 heavy partner if applied to isotopologues of **7** and **11**. Instead, a *p*-phenylenediamine linker, as in ICMAT-3 (**16**)

Scheme 2. Synthesis of Light and Heavy ICMAT-3 pair 16/16*[a]



[a]Yields and positions of ^{13}C labelling in the heavy series are in red. Heavy isotopologues are denoted with * in the text and the Experimental Procedures.

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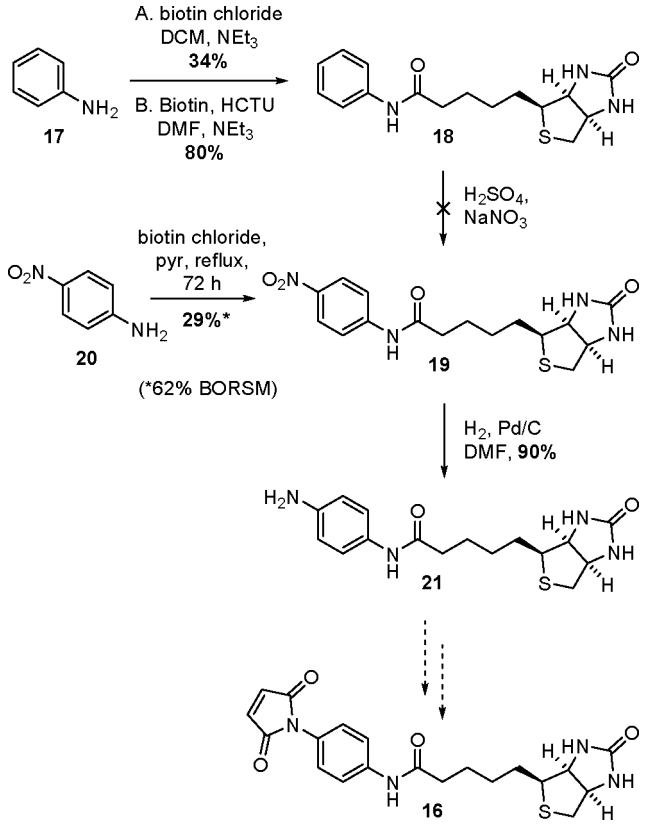
Scheme 3. Synthetic Approach to Light ICMAT-3 16 by Nitration of Biotin Anilide (18) and Coupling of *p*-Nitroaniline (20)



(Scheme 2), was selected, as the heavy version could be synthesized from reasonably priced $^{13}C_6$-aniline.

The light ICMAT-3 (**16**) has been previously synthesized for the purpose of fabricating protein-reactive heteropolytungstate anions as labels for conventional transmission electron microscopy,[30] and as a linker for surface plasmon resonance studies.[31] By varying the order of steps in the previously reported syntheses, we were able to improve on the overall yield of **16** somewhat. As outlined in Scheme 2, ring-opening of maleic anhydride with *p*-phenylenediamine (**12**) gave the maleamic acid **14**.[31] Acylation with biotin chloride then provided the anilide **15**. Finally, cyclization with the peptide coupling agent HCTU (1-[bis(dimethylamino)methylene]-5-chloro-1*H*-benzotriazolium-3-oxide hexafluorophosphate) gave the target **16**. Alternatively, under the action of HCTU, acylation of **14** with biotin was accompanied by cyclization of the maleamic acid moiety to give maleimide **16** in good crude yield. Despite substantial losses during chromatographic purification of **16**, this one-step procedure provided **16** in slightly higher overall yield than the two separate reactions.

While providing the light isotopologue reasonably efficiently, the route outlined in Scheme 2 is suboptimal for application to the heavy congener, because $^{13}C_6$-*p*-phenylenediamine (**12***) is not (readily) commercially available, and its preparation from $^{13}C_6$-aniline requires four steps,[32] doubling the length of the synthesis. Accordingly, other routes were investigated. The first of these is depicted in Scheme 3. Aniline (**17**) was biotinylated, either with biotin chloride or, more straightforwardly and in better yield, with biotin and HCTU. Attempts to nitrate the resulting anilide **18** were unsuccessful. While ^{1}H NMR analysis of the crude reaction products suggested the desired *para* nitration of the anilide moiety had occurred, the signals due to urea NH groups were absent. Although the identity of the product was not confirmed, *N*,*N*′-dinitration of ureas is well-precedented.[33,34]

Efforts were then devoted to the coupling of *p*-nitroaniline (**20**) with biotin (Scheme 3). Unsurprisingly, this non-nucleophilic aniline failed to react with biotin activated by HCTU, despite extended heating. Even the reaction with biotin chloride was sluggish. After extensive attempted optimization, extended heating in pyridine was found to give an acceptable yield of the *p*-nitroanilide **19**, and these conditions allowed the recovery of unreacted *p*-nitroaniline

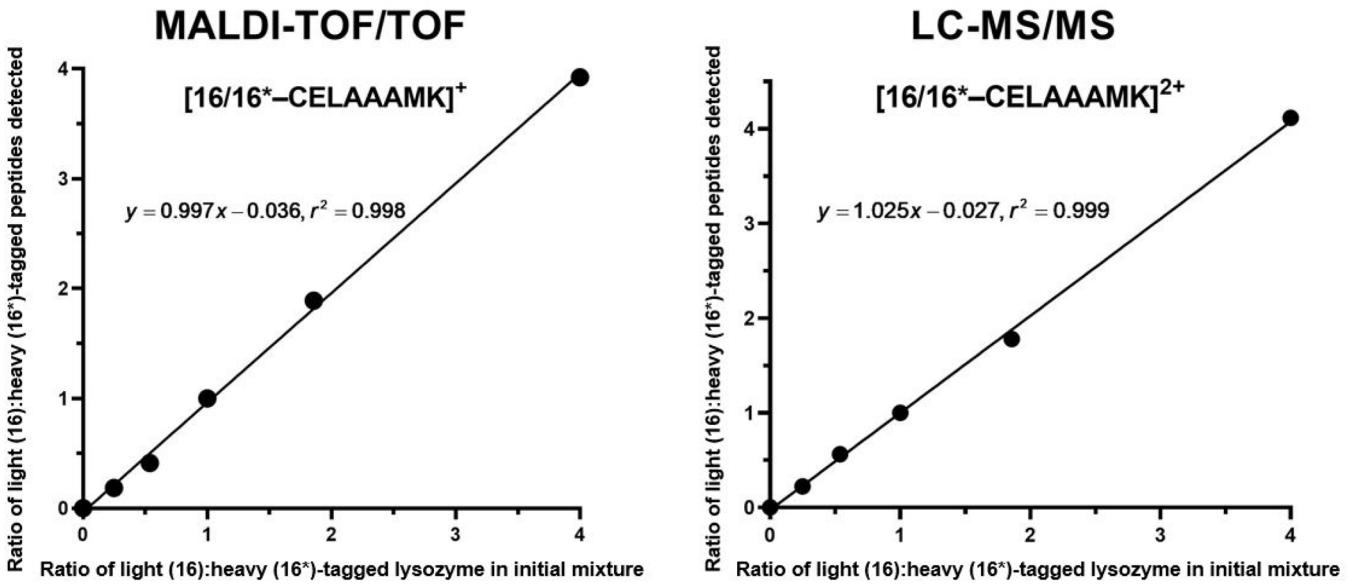


Figure 5. Representative standard curves based on **16**/**16***–CELAAAMK. Ratios of light:heavy-tagged peptides are determined by integral of the [**16**/**16***–CELAAAMK]$^+$ ions in MALDI mass spectra (left) or [**16**/**16***–CELAAAMK]$^{2+}$ extracted-ion chromatogram peak integrals using LC–MS/MS (right).

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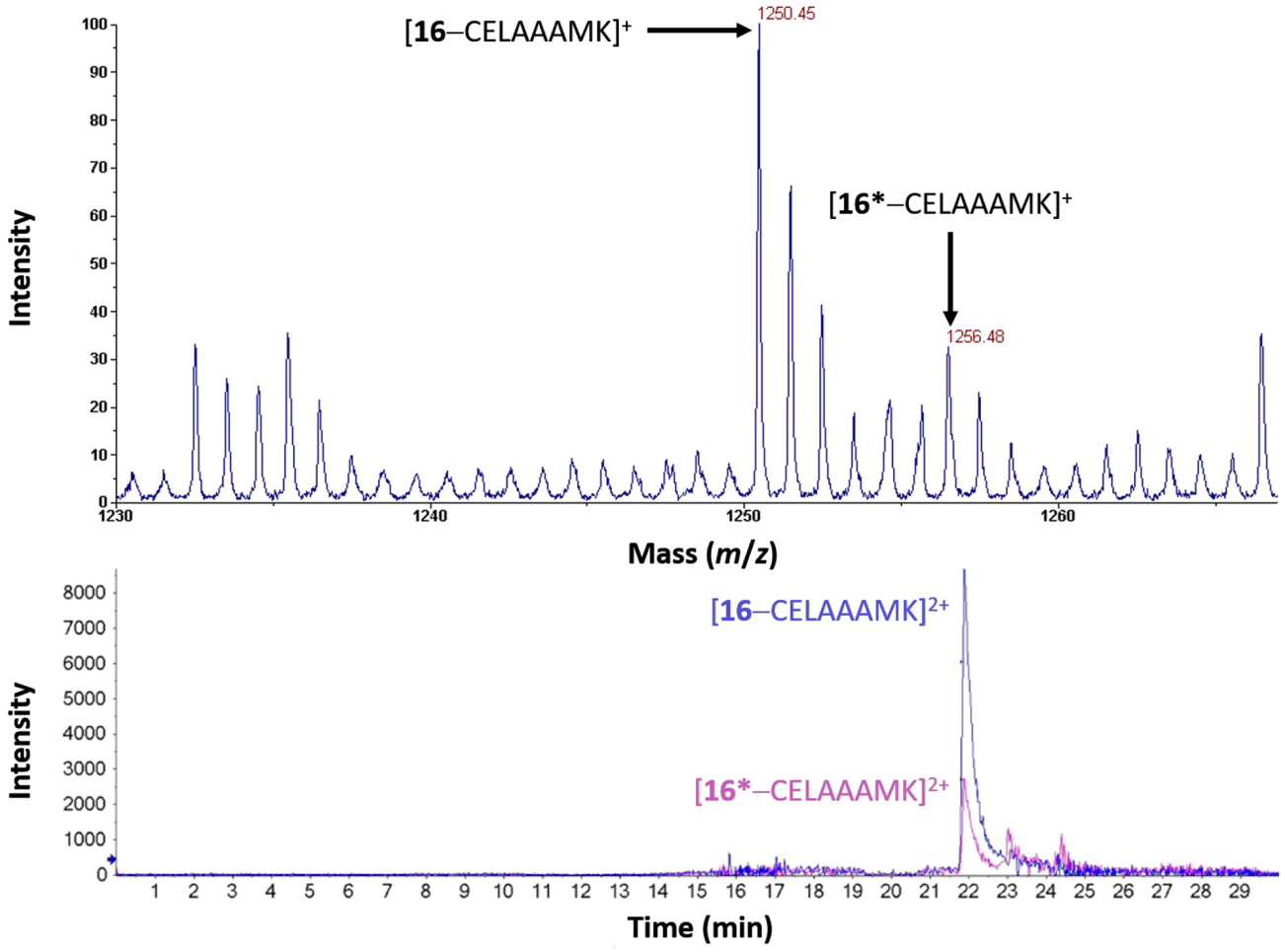

Figure 6. MALDI MS of **16**/**16***–CELAAAMK (top) and an extracted ion chromatogram (ESI+) showing coelution of light- and heavy-tagged peptides (bottom).

(**20**), which would be important for the $^{13}C_6$-labeled version. The coupling conversion, as judged by ^{1}H NMR spectroscopy, was higher in refluxing THF/NEt_3 (60%), but the reaction was messier and these conditions did not allow the recovery of the *p*-nitroaniline. Reduction of the nitro group to give aniline **21** proceeded smoothly with DMF as solvent. An initial attempted reduction in methanol failed to go to completion, assumedly due to the poor solubility of a partially reduced intermediate en route to **21**. Presumably, **21** could have been advanced to ICMAT **16**, but the failure of the nitration of **18** and poor yield of the biotinylation of *p*-nitroaniline (**20**) made these routes unattractive, so this line of investigation was abandoned. Instead, $^{13}C_6$-*p*-phenylenediamine (**12***) was prepared as previously described[32] and coupled with maleimide to give **14*** (Scheme 2). Cyclization/biotinylation then provided the target heavy ICMAT-3 (**16***).

Evaluation of $^{12}C/^{13}C$-Labeled ICMAT-3. As with the deuterated probe, the suitability of the ICMAT-3 pair **16**/**16*** for ratiometric protein quantification was initially assessed using lysozyme. Once again, the ratios of light:heavy-tagged peptide determined by MALDI mass spectrometry, or LC–MS, matched the prepared ratios (Figure 5). A representative MALDI spectrum and extracted-ion chromatogram are also included (Figure 6). Importantly, in the LC–MS trace, perfect coelution of light- and heavy-tagged peptides was observed.

When ICMAT-3 pair **16**/**16*** was used for the analysis of dystrophic dog muscle using a typical proteomic workflow, all light/heavy-tagged peptides were found to coelute. Representative examples of extracted-ion chromatograms of dystrophic dog muscle-derived tagged peptides are shown in Figure 7.

CONCLUSION

Three novel isotope-coded affinity tags bearing pendant maleimide groups, ICMATs-1–3 have been designed, synthesized, characterized, and evaluated in typical proteomics protocols. The electrophilic maleimide moiety has the advantage of more rapid and selective thiol-reactivity than the iodacetamide (IAM) residue in commercially available ICAT tags. Thus, compared to ICATs, smaller excesses of ICMATs are required, reducing non-cysteine conjugation and associated protein misidentification.

ICMAT-1, in which the heavy isotopologue **7*** contains an octadeuterobutylene linker, was shown to be effective for the tagging, identification, and ratiometric analysis of the model protein lysozyme and dystrophic dog muscle tissue proteome. However, some light/heavy-tagged peptide pairs had slightly different HPLC retention times, making this proto/deutero pair nonideal for workflows incorporating a chromatographic fractionation step. In contrast, lysozyme and mammalian muscle-derived peptides tagged with ICMAT-3 (**16***), bearing a $^{13}C_6$-phenylenediamine linker, had identical chromatographic

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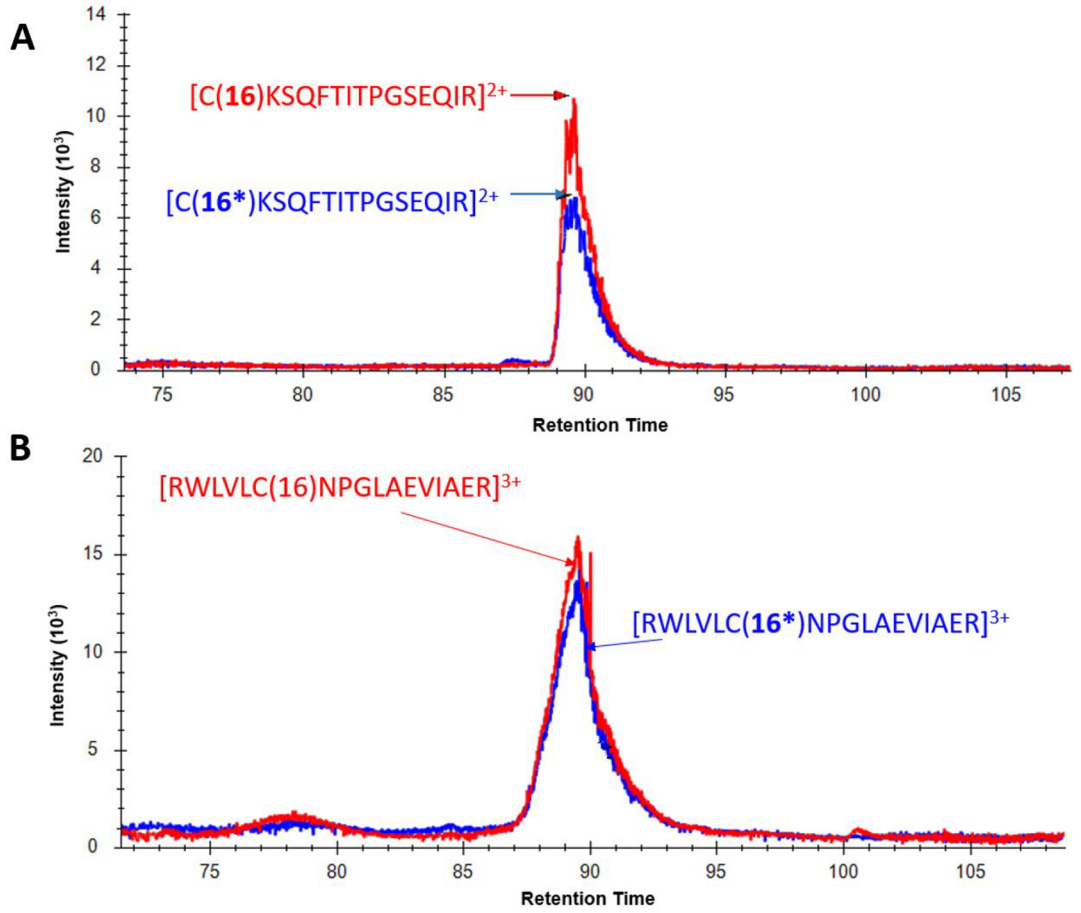

Figure 7. Representative extracted-ion chromatograms of ICMAT-**16**/**16***-tagged peptides from dystrophic dog muscle. (A) Aconitate hydratase-derived peptide [C(**16**/**16***)KSQFTITPGSEQIR]$^{2+}$ with light/heavy m/z values of 847.93 and 850.94, respectively; (B) glycogen phosphorylase-derived peptide [RWLVLC(7/7*)NPGLAEVIAER]$^{3+}$ with light/heavy m/z values of 647.02 and 649.03, respectively.

mobility to the light (**16**-tagged) isotopologues, and retained all the benefits of the deuterated probe. ICMAT-3 has proven useful for our proteomic studies comparing protein−cysteine oxidation state in various normal, stressed, and diseased tissue types.

EXPERIMENTAL PROCEDURES

General. All solvents were distilled prior to use. Anhydrous THF was obtained from a Pure Solv 5-Mid Solvent Purification System (Innovative Technology Inc.). "Dry" DMF, DCM, MeCN, toluene, and MeOH refers to solvents stored over activated 3 Å molecular sieves for at least 24 h. Triethylamine (NEt_3) and pyridine were dried over and distilled from CaH_2 onto KOH pellets under inert gas. All other reagents and materials were purchased from commercial suppliers and used as received.

All reactions were conducted under an inert atmosphere, unless otherwise specified. Where indicated, reaction temperatures refer to the temperature of the heating or cooling bath. All organic extracts were dried over $MgSO_4$, filtered, and evaporated under reduced pressure at 40−50 °C. Trace residual solvent was removed under a stream of N_2 or using high vacuum.

Reaction progress was monitored by TLC using Merck aluminum-backed TLC silica gel 60 F_{254} plates. Spots were visualized directly (colored compounds), by UV light, or by staining with $KMnO_4$ or ninhydrin. Flash column chromatography was performed using Davisil chromatographic silica media LC60A 40−63 μm.

^1H and ^{13}C NMR spectra were acquired using Bruker Avance IIIHD (600 MHz for ^1H and 150 MHz for ^{13}C), Bruker Avance IIIHD (500 MHz for ^1H and 125 MHz for ^{13}C), Varian VNMRS (400 MHz for ^1H and 100 MHz for ^{13}C), or Varian Inova (300 MHz for ^1H and 75 MHz for ^{13}C) spectrometers and calibrated using residual monoprotiated solvent as internal reference.

High-resolution mass spectra were recorded on a Waters Liquid Chromatograph Premier mass spectrometer using ESI or APCI in positive or negative mode, as indicated. IR spectra were recorded on a PerkinElmer Spectrum One FT-IR spectrometer with attenuated total reflectance (ATR) using neat samples.

Synthesis. The protected maleimide **5** was prepared as described previously.[35]

* Denotes heavy isotopologues throughout. Skeleton numbering is shown in the Supporting Information.

*4-Iodobutan-1-ol (**2**).*[25] BF_3•OEt_2 (11.4 g, 80.0 mmol) was added dropwise over 5 min to a solution of NaI (12.0 g, 80.1 mmol) in anhydrous THF (2.88 g, 40.0 mmol) and dry MeCN (80 mL). This solution was stirred overnight, whereupon it developed an orange/brown color. Saturated $NaHCO_3$ (20 mL) was then added, and the reaction mixture was stirred for 30 min before extraction with Et_2O (3 × 30 mL). The extract was washed with water (2 × 20 mL), 1 M sodium thiosulfate

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(30 mL), H_2O (2 × 20 mL), and brine (20 mL), then dried and evaporated to yield the iodide **1** as yellow oil (7.40 g, 93%), which was used without further purification. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 4.84 (br s, 1H, OH), 3.73 (t, J = 6.3 Hz, 2H, OCH_2), 3.23 (t, J = 6.9 Hz, 2H, CH_2I), 1.98−1.86 (m, 2H, CH_2), 1.78−1.66 (m, 2H, CH_2). The ^{1}H NMR data match those reported.[36]

*1,1,2,2,3,3,4,4-Octadeutero-4-iodobutan-1-ol (**2***).[37] Prepared in a similar fashion to **1**, using d_8-THF (0.80 g, 10.0 mmol), BF_3•OEt_2 (2.84 g, 20.0 mmol), and NaI (3.00 g, 20.0 mmol) in dry MeCN (20 mL). The iodide **2*** was isolated as a yellow oil (2.00 g, 96%). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.62 (br s, 1H, OH + H_2O).

*4-Iodobutyl 5-((4R)-2-oxohexahydro-1H-thieno[3,4-d]-imidazol-4-yl)pentanoate (4-iodobutyl D-biotinate) (**4**).* The method of Jung et al. was adapted.[38] A dry round-bottom flask was fitted with a Dean−Stark apparatus and charged with D-(+)-biotin (**3**) (0.98 g, 3.9 mmol), TsOH (0.14 g, 0.80 mmol), and dry toluene (70 mL) under argon. The stirred mixture was heated to 50 °C, and (**1**) (1.60 g, 8.00 mmol) was added over 5 min. The solution was heated under reflux for 24 h, then the toluene was evaporated to give a brown residue, which was subjected to flash chromatography. Elution with DCM then 1:9 MeOH/DCM gave ester **4** as a yellow solid (0.75 g, 45%), mp = 94−96 °C. IR (thin film) cm^{-1}: 3214 (br, 2 × NH), 1727 (O═CO) 1697 (C═O). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 5.37 (br s, 1H, NH), 5.03 (br s, 1H, NH), 4.54−4.49 (m, 1H, H6a′), 4.34−4.30 (m, 1H, H3a′), 4.10 (t, J = 6.4 Hz, 2H, H1″), 3.21 (t, J = 6.8 Hz, 2H, H4″), 3.20−3.14 (m, 1H, H4′), 2.96 (dd, J = 4.8, 12.8 Hz, 1H, H6′), 2.74 (d, J = 12.8 Hz, 1H, H6′), 2.33 (t, J = 7.6 Hz, 2H, H2), 1.94−1.86 (m, 2H, CH_2, H2″), 1.78−1.64 (m, 6H, 3 × CH_2, H3, 5, 3″), 1.50−1.38 (m, 2H, CH_2, H4). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 173.7 (C1), 163.3 (C2′), 63.4 (C1″), 62.1 (C3a′), 60.2 (C6a′), 55.4 (C4′), 40.7 (C6′), 34.0 (C2), 30.2, 29.7, 28.5, 28.4, 24.9, 6.1 (C4″). HRMS (ESI+) m/z: Calcd for $C_{14}H_{23}IN_2NaO_3S^+$ [M + Na]$^+$, 449.0366; found, 449.0362.

*1,1,2,2,3,3,4,4-Octadeutero-4-iodobutyl 5-((4R)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanoate (1,1,2,2,3,3,4,4-octadeutero-4-iodobutyl D-biotinate) (**4***).* **4*** was prepared as described for **4** from **2*** (800 mg, 4.00 mmol), D-(+)-biotin (**3**) (488 mg, 2.00 mmol), and TsOH (70.0 mg 0.20 mmol), and yielding **4*** as a yellow powder (0.588 g, 68%), mp = 94−96 °C. IR (thin film) cm^{-1}: 3211 (br, 2 × NH), 1726 (OC═O), 1697 (C═O). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 5.63 (br s, 1H, NH), 5.26 (br s, 1H, NH), 4.52−4.49 (m, 1H, CH, H6a′), 4.33−4.30 (m, 1H, CH, H3a′), 3.18−3.13 (m, 1H, H4′), 2.94 (dd, J = 5.1, 12.8 Hz, 1H, H6′), 2.74 (d, J = 12.8 Hz, 1H, H6′), 2.33 (t, J = 7.6 Hz, 2H, H2), 1.75−1.64 (m, 4H, H3, 5), 1.50−1.38 (m, 2H, CH_2, H4). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 173.8 (C1), 163.6 (C2′), 62.1 (C3a′), 60.2 (C6a′), 55.5 (C4′), 40.7 (C6′), 34.0 (C2), 28.5, 28.4, 24.9. HRMS (ESI+) m/z: Calcd for $C_{14}H_{15}D_8IN_2NaO_3S^+$ [M + Na]$^+$, 457.0868; found, 457.0872.

*(4-(4,7-Epoxy-1,3-dioxo-3a,4-dihydro-1H-isoindol-2-(3H,7H,7aH)-yl)butyl D-biotinate (**6**).* This method was adapted from that of Reetz et al.[39] A solution of iodide **4** (0.50 g, 1.2 mmol) in anhydrous DMF (5 mL) was added dropwise over 2 min to a stirred suspension of **5**[35] (0.231 g, 1.40 mmol) and K_2CO_3 (0.581 g, 4.20 mmol) in anhydrous DMF (10 mL) at 50 °C under N_2. The reaction mixture turned yellow initially before developing a red/brown color. After 4 h, the reaction mixture was diluted with H_2O (150 mL) and extracted into EtOAc (4 × 30 mL). The extract was washed with H_2O (2 × 20 mL) and brine (20 mL), dried, and evaporated. The brown residue was subjected to flash chromatography. Elution with a gradient of DCM to 1:9 MeOH/DCM yielded **6** as a white powder (102 mg, 55%), mp = 146−148 °C. IR (thin film) cm^{-1}: 3320 (NH), 3212 (br, NH), 1723 (OC═O), 1703 (C═O), 1683 (C═O), 1657 (C═O). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 6.51 (s, H5‴/6‴), 5.48 (br s, 1H, NH), 5.27 (s, 2H, H4‴/7‴), 4.99 (br s, 1H, NH), 4.53−4.49 (m, 1H, CH, H6a′), 4.34−4.30 (m, 1H, CH, H3a′), 4.06 (m, 2H, H1″), 3.52 (m, 2H, H4″), 3.20−3.12 (m, 1H, H4′), 2.92 (dd, J = 5.1 Hz, 12.9 Hz, H6′), 2.86 (s, 2H, H3a‴/7a‴), 2.72 (d, J = 13.2 Hz, 1H, H6′), 2.32 (t, J = 6.9 Hz, 2H, H2), 1.80−1.60 (m, 8H, 4 × CH_2), 1.50−1.38 (m, 2H, H4). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 176.6 (C1‴/3‴), 173.8 (C1), 163.3 (C2′), 136.7 (C5‴/6‴), 81.1 (C4‴/7‴), 63.8 (C1″), 62.0 (C3a′), 60.2 (C6a′), 55.4 (C4′), 47.6 (C3a‴/7a‴), 40.7 (C6′), 38.6, 34.0 (C2), 28.4, 26.0, 25.9, 24.9, 24.4. HRMS (ESI+) m/z: Calcd for $C_{22}H_{29}N_3NaO_6S^+$ [M + Na]$^+$, 486.1669; found, 486.1674.

*1,1,2,2,3,3,4,4-Octadeutero- (4-(4,7-epoxy-1,3-dioxo-3a,4-dihydro-1H-isoindol-2(3H,7H,7aH)-yl)butyl D-biotinate (**6***).* This compound was prepared as described for **6** using iodide **4*** (300 mg, 0.70 mmol), **5**[35] (165 mg, 1.00 mmol), K_2CO_3 (420 mg, 3.00 mmol), and anhydrous DMF (7 mL), yielding **6*** as a white solid (148 mg, 48%), mp = 144−146 °C. IR (thin film) cm^{-1}: 3322 (NH), 3219 (br, NH), 1702 (C═O), 1680 (C═O), 1658 (C═O). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 6.51−6.52 (m, 2H, C5‴/6‴), 5.77 (br s, 1H, NH), 5.30 (br s, 1H, NH), 5.26 (s, 2H, H7‴, 4‴, 4.52−4.48 (m, 1H, CH, H6a′), 4.33−4.29 (m, 1H, CH, H3a′), 3.16−3.12 (m, 1H, H4′), 2.93 (dd, J = 4.8, 12.9 Hz, 1H, H6′), 2.86 (s, 2H, H3a‴/7a‴), 2.72 (d, J = 12.9 Hz, 1H, H6′), 2.31 (t, J = 7.2 Hz, 2H, H2), 1.80−1.60 (m, 4H, H3, 5), 1.48−1.41 (m, 2H, H4). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 176.6, (C1‴/3‴), 173.8 (C1), 163.6 (C2′), 136.7 (C5‴/6‴), 81.1 (C4‴/7‴), 62.0 (C3a′), 60.2 (C6a′), 55.5 (C4′), 47.6 (C3a‴/7a‴), 40.7 (C6′), 34.0 (C2), 28.4, 28.3, 24.9. HRMS (ESI+) m/z: Calcd for $C_{22}H_{21}D_8N_3NaO_6S^+$ [M + Na]$^+$, 494.2171; found, 494.2170.

*4-(2,5-Dioxo-2,5-dihydro-1H-pyrrol-1-yl)butyl 5-((4R)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanoate (**7**).* Following a variation of the method of Reetz et al.,[39] **6** (225 mg, 0.485 mmol) was dissolved in dry toluene (50 mL) and heated under reflux for 24 h. The solution was evaporated, and the white solid residue was subjected to flash chromatography. Elution with DCM−1:9 MeOH:DCM yielded **7** as a white powder (141 mg, 74%), mp = 90−92 °C. IR (thin film) cm^{-1}: 3255 (br NH), 1698 (C═O, unresolved). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 6.71 (s, 2H, H4‴/5‴), 5.82 (br s, 1H, NH), 5.40 (br s, 1H, NH), 4.50−4.48 (m, 1H, H6a′), 4.34−4.30 (m, 1H, H3a′), 4.07 (t, J = 6.4 Hz, 2H, H1″), 3.54 (t, J = 6.8 Hz, 2H, H4‴), 3.20−3.12 (m, 1H, H4′), 2.90 (dd, J = 4.8 Hz, J = 12.6 Hz, 1H, H6′), 2.72 (d, J = 12.6 Hz, 1H, H6′), 2.31 (t, J = 7.6 Hz, 2H, H2), 1.78−1.64 (m, 6H), 1.50−1.38 (m, 2H, H4). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 173.8 (C1), 171.0 (C1‴/3‴), 163.7 (C2′), 134.3 (C4‴/5‴), 63.8 (C1″), 62.1 (C3a′), 60.2 (C6a′), 55.5 (C4′), 40.7 (C6′), 37.5 (C4″), 34.0 (C2), 28.5, 28.3, 26.0, 25.3, 24.9. HRMS (ESI+) m/z: Calcd for $C_{18}H_{25}N_3O_5SNa^+$ [M + Na]$^+$, 418.1407; found, 418.1411.

*1,1,2,2,3,3,4,4-Octadeutero-4-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)butyl 5-((4R)-2-oxohexahydro-1H-thieno[3,4-d]-imidazol-4-yl)pentanoate (**7***).* Following the procedure for

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the synthesis of **7**, using **6*** (225 mg, 0.477 mmol) gave **7*** as a white powder (167 mg, 84%), mp = 88−90 °C. IR (thin film) cm^{-1}: 3222 (br, NH), 1690 (C═O unresolved). ^1H NMR ($CDCl_3$, 300 MHz): δ 6.70 (s, 2H, H4‴, 5‴), 5.97 (br s, 1H, NH), 5.58 (br s, 1H, NH), 4.51−4.47 (m, 1H, CH, H6a′), 4.32−4.27 (m, 1H, CH, H3a′), 3.20−3.11 (m, 1H, CH, H4′), 2.92 (dd, J = 4.8 Hz, J = 12.9 Hz, 1H, H6′), 2.72 (d, J = 12.9 Hz, 1H, H6′), 2.30 (t, J = 7.2 Hz, 2H, H2), 1.78−1.60 (m, 4H), 1.50−1.38 (m, 2H, CH_2, H4). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 173.7 (C1), 171.0 (C1‴/3‴), 163.1 (C2′), 134.3 (C4‴/5‴), 62.0 (C3a′), 60.2 (C6a′), 55.3 (C4′), 40.7 (C6′), 34.0 (C2), 28.4, 28.4, 24.9. HRMS (ESI+) m/z: Calcd for $C_{18}H_{17}D_8N_3O_5SNa^+$ $[M + Na]^+$, 426.1909; found, 426.1927.

*4-Azidobutyl 5-((4R)-2-Oxohexahydro-1H-thieno[3,4-d]-imidazol-4-yl)pentanoate (**8**).* Following a method adapted from Bates et al.,[40] **4** (213 mg, 0.500 mmol) was dissolved in anhydrous DMF (5 mL) at 0 °C. NaN_3 (100 mg, 1.50 mmol) was added portion-wise, and the resultant suspension was allowed to warm to room temperature and stirring was continued for 24 h. The solution was diluted with H_2O (150 mL) and extracted with $CHCl_3$ (4 × 30 mL). The extract was washed with water (2 × 20 mL) and brine (20 mL), dried, and evaporated, and the residue was subjected to flash chromatography. Elution with DCM−1:9 MeOH/DCM yielded **8** as a yellow waxy solid (160 mg, 94%), mp = 86 °C. IR (thin film) cm^{-1}: 3216 (NH), 2095 (N_3), 1727 (C═O), 1697 (C═O). ^1H NMR ($CDCl_3$, 400 MHz): δ 5.77 (br s, 1H, NH), 5.39 (br s, 1H, NH), 4.53−4.48 (m, 1H, CH, H6a′), 4.33−4.29 (m, 1H, CH, H3a′), 4.09 (t, J = 6.4 Hz, 2H, 1H″), 3.32 (t, J = 6.8 Hz, 2H, H4″), 3.20−3.12 (m, 1H, H4′), 2.90 (dd, J = 4.8 Hz, J = 12.8 Hz, 1H, H6′), 2.73 (d, J = 12.8 Hz, 1H, H6′), 2.33 (t, J = 7.6 Hz, 2H, H2), 2.00−1.86 (m, 2H, CH_2), 1.76−1.62 (m, 6H), 1.50−1.38 (m, 2H, CH_2, H4). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 173.9 (C1), 163.7 (C2′), 63.8 (C1″), 62.1 (C3a″), 60.2 (C6a′), 55.5 (C4′), 51.1, 40.7, 34.0, 28.5, 28.4, 26.0, 25.7, 24.9. HRMS (ESI+) m/z: Calcd for $C_{14}H_{23}N_5O_3SNa^+$ $[M + Na]^+$, 364.1414; found, 364.1420.

*1,1,2,2,3,3,4,4-Octadeutero-4-azidobutyl 5-((4R)-2-oxo-hexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanoate (**8***).* A procedure similar to that above was applied to **4*** (294 mg, 0.677 mmol) and NaN_3 (137 mg, 2.10 mmol) in anhydrous DMF (5 mL) to afford **8*** as a yellow waxy solid (214 mg, 90%), mp = 84−86 °C. IR (thin film) cm^{-1}: 3215, 2096 (N_3), 1728 (C═O), 1699 (C═O). ^1H NMR ($CDCl_3$, 400 MHz): δ 5.65 (br s, 1H, NH), 5.28 (br s, 1H, NH), 4.52−4.49 (m, 1H, CH, H6a′), 4.32−4.29 (m, 1H, CH, H3a′), 3.18−3.13 (m, 1H, H4′), 2.91 (dd, J = 4.8 Hz, J = 12.8 Hz, 1H, H6′), 2.73 (d, J = 12.8 Hz, 1H, H6′), 2.33 (t, J = 7.6 Hz, 2H, H2), 1.76−1.63 (m, 4H), 1.50−1.38 (m, 2H, CH_2, H4). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 173.8 (C1), 163.6 (C2′), 62.1 (C3a′), 60.2 (C6a′), 55.5 (C4′), 40.7 (C6′), 34.0 (C2), 28.5, 28.4, 24.9. HRMS (ESI+) m/z: Calcd for $C_{14}H_{15}D_8N_5O_3SNa^+$ $[M + Na]^+$, 372.1916; found, 372.1924.

*2-(2-Propynyl)-3a,4,7,7a-tetrahydro-4,7-epoxy-1H-isoin-dole-1,3(2H)-dione (**9**).* This method was adapted from Reetz et al.[39] A solution of propargyl bromide (0.55 g, 4.6 mmol) in anhydrous DMF (10 mL) was added dropwise to a suspension of the protected maleimide (**5**)[35] (0.39 g, 2.3 mmol) and K_2CO_3 (1.60 g, 11.5 mmol) in anhydrous DMF (10 mL) under argon. The reaction mixture was heated to 50 °C and stirred for 6 h. It was then diluted with H_2O (400 mL) and extracted with EtOAc (4 × 30 mL). The extract was washed with water (2 × 20 mL) and brine (20 mL), dried, and evaporated, and the residue was subjected to flash chromatography. Elution with 1:1 EtOAc/hexanes gave **12** as a white solid (180 mg, 39%). ^1H NMR (d_6-DMSO, 400 MHz): δ 6.53 (d, J = 0.9 Hz, 2H, HC═CH), 5.31 (d, J = 0.6 Hz, 2H, HCO), 4.24 (d, J = 2.4 Hz, 2H, αCH), 2.91 (s, 2H, $H_2CC\equiv$), 2.20 (s, 1H, ≡CH). The ^1H NMR data match those reported.[39]

*4-(4-((4,7-Epoxy-1,3-dioxo-3a,4-dihydro-1H-isoindol-2-(3H,7H,7aH)-yl)methyl)-1H-1,2,3-triazol-1-yl)butyl D-biotinate (**10**).* This was adapted from the method of Mukai et al.[41] The masked *N*-propargylmaleimide **9** (142 mg, 0.699 mmol) was added to a stirred solution of $CuSO_4{\cdot}5H_2O$ (15 mg, 0.060 mmol) and sodium ascorbate (24 mg, 0.12 mmol) in 1:1 DMF:H_2O (5 mL). After 5 min, **8** (171 mg, 0.501 mmol) was added, whereupon a deep red color developed. After 24 h, the reaction mixture was diluted with H_2O (200 mL) and extracted with EtOAc (6 × 20 mL). The extract was washed with water (20 mL) and brine (3 × 20 mL), dried, and evaporated, and the residue was subjected to flash chromatography. Elution with DCM then 1:9 MeOH:DCM gave **13** as a white solid (70 mg, 28%), mp = 150−152 °C. IR (thin film) cm^{-1}: 3245 (br, NH), 1695 (C═O unresolved). ^1H NMR ($CDCl_3$, 300 MHz): δ 7.50 (s, 1H, H5‴), 6.53 (s, 2H, H5′‴/6′‴), 5.34 (br s, 1H, NH), 5.29 (s, 2H, H4′‴/7′‴), 4.94 (br s, 1H, NH), 4.79 (s, 2H, Hα4‴), 4.54−4.50 (m, 1H, CH, H6a′), 4.34 (t, J = 6.9 Hz, 2H, H4″), 4.34 (m, 1H, H3a′) 4.08 (t, J = 6.3 Hz, 2H, H1″), 3.20−3.12 (m, 1H, H4′), 2.92 (dd, J = 5.1 Hz, J = 12.6 Hz, 1H, H6′), 2.91 (s, 2H, H3a′‴/7a′‴), 2.72 (d, J = 12.9 Hz, 1H, H6′), 2.32 (t, J = 7.2 Hz, 2H, H2), 2.00−1.90 (m, 2H), 1.80−1.60 (m, 6H, CH_2), 1.50−1.38 (m, 2H, H4). ^{13}C NMR (CDCl3, 75 MHz): δ 175.9 (C1′‴/3′‴), 173.7 (C1), 163.2 (C2′), 142.4, 136.7 (C5′‴/6′‴), 122.6, 81.2 (C4′‴/7′‴), 63.5 (C1″), 62.1 (C3a′), 60.2 (C6a′), 55.4 (C4′), 50.0 47.7 (C3a′‴/7a′‴), 40.7, 34.3, 34.0, 28.55, 28.4, 27.2, 25.8, 24.9. HRMS (ESI+) m/z: Calcd for $C_{25}H_{32}N_6O_6SK^+$ $[M +K]^+$, 583.1736; found, 583.1724.

*4-(4-((1,3-Dioxo-3a,4,7,7a-tetrahydro-1H-4,7-epoxyisoin-dol-2(3H)-yl)methyl-1H-1,2,3-tirazol-1-ylbutyl biotinate (**10***).* The procedure above was applied to **8*** (191 mg, 0.547 mmol), **9** (176 mg, 0.866 mmol), $CuSO_4{\cdot}5H_2O$ (13 mg, 0.052 mmol) and sodium ascorbate (20 mg, 0.10 mmol) in 1:1 DMF:H_2O (5 mL). Purification as above gave **10*** as a white powder (129 mg, 47%), mp = 148−150 °C. IR (thin film) cm^{-1}: 3312 (br, NH), 1742 (C═O), 1698 (C═O). ^1H NMR ($CDCl_3$, 300 MHz): δ 7.49 (s, 1H, H5‴), 6.50 (s, 2H, H5′‴/6′‴), 5.99 (br s, 1H, NH), 5.58 (br s, 1H, NH), 5.26 (s, 2H, H4′‴/7′‴), 4.75 (s, 2H, CH_2, Hα4‴), 4.50−4.46 (m, 1H, CH, H6a′), 4.30−4.26 (m, 1H, CH, H3a′), 3.14−3.12 (m, 1H, H4′), 2.89 (dd, J = 7.5 Hz, J = 19.5 Hz, 1H, H6′), 2.89 (s, 2H, H3a′‴/7a′‴), 2.70 (d, J = 12.9 Hz, 1H, H6′), 2.29 (t, J = 7.5 Hz, 2H, H2), 1.80−1.60 (m, 4H, CH_2), 1.46−1.38 (m, 2H, CH_2, H4). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 175.8, (C1′‴/3′‴), 173.8 (C1), 163.8 (C2′), 142.3, 136.7 (C5′‴/6′‴), 122.6, 81.1 (C4′‴/7′‴), 62.0 (C3a′), 60.2 (C6a′), 55.6 (C4′), 47.6 (C3a′‴/7a′‴), 40.6, 34.3, 34.0, 28.56, 28.3, 24.9. HRMS (ESI +) m/z: Calcd for $C_{25}H_{24}D_8N_6O_6SNa^+$ $[M + Na]^+$, 575.2498; found, 575.2510.

*4-(4-((2,5-Dioxo-2,5-dihydro-1H-pyrrol-1-yl)methyl)-1H-1,2,3-triazol-1-yl)butyl biotinate (**11**).* A solution of **10** (70 mg, 0.15 mmol) in dry toluene (20 mL) was heated under reflux for 24 h. The volatiles were evaporated, and the residue was subjected to flash chromatography. Elution with DCM−1:9 MeOH:DCM gave **11** as a white powder (49 mg, 81%), mp = 144 °C. IR (thin film) cm^{-1}: 3227 (br, NH), 1700 (C═

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O unresolved), 1646 (C═O). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.56 (s, 1H, H5‴), 6.74 (s, 2H, H4′‴/5′‴), 5.11 (br s, 1H, NH), 4.82 (s, 2H, Hα4″), 4.79 (br s, 1H, NH), 4.54−4.50 (m, 1H, CH, H6a′), 4.36 (t, J = 7.2 Hz, 2H, H4″), 4.35−4.32 (m, 1H, CH, H3a′), 4.09 (t, J = 6.4 Hz, 2H, CH2, H1″), 3.20−3.15 (m, 1H, H4′), 2.95 (dd, J = 4.8 Hz, J = 12.8 Hz, 1H, H6′), 2.73 (d, J = 12.8 Hz, 1H, H6′), 2.33 (t, J = 7.6 Hz, 2H, H2), 2.01−1.94 (m, 2H), 1.74−1.59 (m, 6H, CH_2), 1.50−1.42 (m, 2H, CH_2, H4). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 173.6 (C1′‴/3′‴), 170.3 (C1), 163.0 (C2′), 142.8, 134.5 (C4′‴/5′‴), 122.8, 63.58 (C1″), 62.0 (C3a′), 60.2 (C6a′), 55.4 (C4′), 50.0, 40.7, 33.9, 33.0, 28.4, 28.4, 27.2, 25.8, 24.9. HRMS (ESI+) m/z: Calcd for $C_{21}H_{28}N_6O_5SNa^+$ $[M + Na]^+$, 499.1734; found, 499.1746.

*1,1,2,2,3,3,4,4-Octadeutero-4-(4-((2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)methyl)-1H-1,2,3-triazol-1-yl)butyl biotinate (**11***).* The procedure above was applied to **10*** (110 mg, 0.20 mmol) in dry toluene (10 mL). Purification as above gave **11*** as a white powder (83 mg, 87%), mp = 150−152 °C. IR (thin film) cm^{-1}: 3228 (br, NH), 1700 (C═O), 1684 (C═O), 1646 (C═O). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.55 (s, 1H, CH, H5‴), 6.74 (s, 2H, H4′‴/5′‴), 5.19 (br s, 1H, NH), 4.86 (br s, 1H, NH), 4.82 (s, 2H, Hα4′), 4.54−4.51 (m, 1H, CH, H6a′), 4.34−4.31 (m, 1H, CH, H3a′), 3.20−3.14 (m, 1H, H4′), 2.93 (dd, J = 5.2 Hz, J = 12.8 Hz, 1H, H6′), 2.73 (d, J = 12.8 Hz, 1H, H6′), 2.33 (t, J = 7.2 Hz, 2H, H2), 1.75−1.59 (m, 4H, CH_2), 1.50−1.42 (m, 2H, CH_2, H4). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 173.7 (C1′‴/3′‴), 170.3 (C1), 163.1 (C2′), 142.8, 134.5 (C4′‴/5′‴), 122.8, 62.1 (C3a′), 60.2 (C6a′), 55.4 (C4′), 40.7, 33.9, 33.0, 28.4, 28.4, 24.9. HRMS (ESI+) m/z: Calcd for $C_{21}H_{20}D_8N_6O_5SNa^+$ $[M + Na]^+$, 507.2236; found, 507.2242.

*$^{13}C_6$-p-Phenylenediamine (**12***).* A suspension of 10% Pd/C (72 mg) in a solution of $^{13}C_6$-*p*-nitroaniline (**20***)[42] (75 mg, 0.52 mmol) in dry MeOH (2 mL) was stirred under a balloon of H_2 for 5 h, after which time TLC (EtOAc) indicated the reaction was complete. The mixture was diluted with DCM (10 mL) and filtered through Celite. The solvent was evaporated to afford **12*** as pale pink crystals (51 mg, 86%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 6.33−6.85 (m, 4H, ArH), 3.35 (s, 4H, NH_2). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 138.1−139.1 (m, CH), 116.4−116.9 (m, CNH_2). HRMS (ESI+) m/z: calcd. for $^{13}C_6H_9N_2^+$ $[M + H]^+$ 115.0962, found 115.0960.

*(Z)-3-((4-Aminophenyl)carbamoyl)prop-2-enoic acid (N-4-aminophenylmaleamic acid) (**14**).*[31] A solution of maleic anhydride (0.98 g, 10 mmol) in anhydrous THF (10 mL) was added dropwise to a stirred solution of *p*-phenylenediamine (**12**) (1.08 g, 10.0 mmol) in anhydrous THF (20 mL) over 30 min, whereupon a precipitate formed. After 24 h, the suspension was vacuum filtered and the precipitate washed with DCM (30 mL). The olive-green powder was triturated with boiling MeOH (50 mL) and collected by vacuum filtration to give the maleamic acid **14** as a gray powder (1.85 g, 90%). ^{1}H NMR (d_6-DMSO, 400 MHz): δ 10.53 (s, 1H, NH), 7.29 (d, J = 8.8 Hz, 2H, H2′/6′), 6.53 (d, J = 8.8 Hz, 2H, H3′/5′), 6.46 (d, J = 12.4 Hz, 1H, H2), 6.28 (d, J = 12.4 Hz, 1H, H3), NH_2 and COOH not observed. The ^{1}H NMR data match those reported.[31]

*(Z)-3-((4-Amino(1,2,3,4,5,6-$^{13}C_6$)phenyl)carbamoyl)prop-2-enoic acid (N-4-amino(1,2,3,4,5,6-$^{13}C_6$)phenylmaleamic acid) (**14***).* The procedure above was applied to solutions of **12*** (51 mg, 0.45 mmol) in EtOAc (3 mL) and maleic anhydride (44 mg, 0.45 mmol) in EtOAc (1 mL). Purification by rapid silica filtration (1:19−1:10 MeOH/DCM) gave **14*** as an off-white powder (55 mg, 58%). ^{1}H NMR (d_6-DMSO, 500 MHz): δ 10.64 (s, 1H, NH), 7.30 (dm, J = 158.3 Hz, 2H, H2′/6′), 6.52 (dm, J = 154.8 Hz, 2H, H3′/5′), 6.45 (d, J = 12.4 Hz, 1H, H2), 6.25 (d, J = 12.4 Hz, 1H, H3), NH_2 and COOH not observed. ^{13}C NMR (d_6-DMSO, 100 MHz): δ 166.2 (C4), 162.5 (C1), 146.0 (td, J_1 = 60.5 Hz, J_2 = 8.9 Hz, C1′), 132.3 (C2), 131.8 (d, J = 3.7 Hz, C3), 126.8 (td, J_1 = 63.9 Hz, J_2 = 8.8 Hz, C4′), 121.4 (td, J_1 = 61.8 Hz, J_2 = 5.4 Hz, C2′/6′), 113.7 (td, J_1 = 60.2 Hz, J_2 = 5.8 Hz, C3′/5′).

*(Z)-4-Oxo-4-((4-(((5-((3aR,4R,6aS)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanoyl)oxy)amino)phenyl)-amino)but-2-enoic acid (N-4-(D-biotinylaminophenyl)-maleamic acid) (**15**).* D-(+)-Biotin (**3**) (245 mg, 1.00 mmol) was stirred with $SOCl_2$ (1.5 mL) under a $CaCl_2$ guard tube for 2 h, after which the $SOCl_2$ was evaporated under a stream of N_2. The residue was dissolved in THF (5 mL) and DMF (3 drops). Aniline **14** (206 mg, 1.00 mmol) and NEt_3 (0.28 mL, 2.0 mmol) were dissolved in anhydrous DMF (2.5 mL) and cooled to 0 °C. The biotin chloride solution was added dropwise to the aniline solution over 40 min. The solvent was evaporated, and the residue was diluted with H_2O (20 mL) added. The resulting precipitate was collected and triturated with boiling EtOH, affording a yellow−green powder (246 mg, 57%). ^{1}H NMR (d_6-DMSO, 400 MHz): δ 11.17 (br s, 1H, NH), 9.85 (s, 1H, NH), 7.53 (s, 4H, ArH), 6.43 (s, 1H, NH), 6.21−6.36 (m, 3H, NH + H2/3), 4.28−4.32 (m, 1H, CH, H6a′), 4.12−4.16 (m, 1H, CH, H3a′), 3.10−3.14 (m, 1H, CH, H4′), 2.82 (dd, J_1 = 5.1 Hz, J_2 = 12.4 Hz, 1H, H6′), 2.58 (d, J = 12.4 Hz, 1H, H6′), 2.33 (t, J = 8.0 Hz, 2H, CH_2, H2″), 1.33−1.69 (m, 6H, 3 × CH_2, H3″/4′′/5′′).

*1-(4-(((5-((3aR,4R,6aS)-2-Oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanoyl)oxy)amino)phenyl)-1H-pyrrole-2,5-dione (N-4-(D-biotinylaminophenyl)maleimide) (**16**).* Method A (Adapted from Quideau et al.[31]): HCTU (63 mg, 0.15 mmol) was added to a stirred solution of the maleamic acid **15** (42 mg, 0.098 mmol) and Hunig's base (26 mg, 0.20 mmol) in anhydrous DMF (5 mL) at 0 °C, under N_2. The solution was allowed to warm to room temperature. After 4.5 h, the reaction was poured into water and extracted with EtOAc (3 × 20 mL). The combined organic phase was washed with H_2O (20 mL), dilute HCl (20 mL), $NaHCO_3$ (20 mL), and brine (20 mL). The solution was dried and evaporated, and the residue was subjected to flash chromatography. Elution with 1:19 MeOH/DCM gave the maleimide **16** as a pale yellow powder (18 mg, 43%). ^{1}H NMR (d_6-DMSO, 500 MHz) δ 10.03 (s, 1H, NH), 7.67 (d, J = 8.9 Hz, 2H, H2‴/6‴), 7.23 (d, J = 8.9 Hz, 2H, H3‴/5‴), 7.16 (s, 2H, H3/4) 6.43 (s, 1H, NH), 6.35 (s, 1H, NH), 4.28−4.32 (m, 1H, CH, H6a′), 4.12−4.16 (m, 1H, CH, H3a′), 3.10−3.14 (m, 1H, CH, H4′), 2.82 (dd, J_1 = 5.1 Hz, J_2 = 12.4 Hz, 1H, H6′), 2.58 (d, J = 12.4 Hz, 1H, H6′), 2.33 (t, J = 8.0 Hz, 2H, CH_2, H2″), 1.33−1.69 (m, 6H, 3 × CH_2, H3″/4″/5″). The ^{1}H NMR data match those reported.[31]

Method B: Maleamic acid **14** (202 mg, 0.980 mmol) was added to a stirred, dark yellow solution of D-(+)-biotin (**3**) (250 mg, 1.02 mmol), HCTU (1.28 g, 3.09 mmol), and Hünig's base (0.7 mL, 4 mmol) in anhydrous DMF (7 mL), upon which the solution turned dark red. After stirring overnight, the reaction mixture was diluted with water (100 mL) to afford a gray precipitate, which was collected by vacuum filtration, then dissolved in 1:9 MeOH:DCM (100

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mL). The solution was washed with 5% $NaHCO_3$ (3 × 100 mL) and water (100 mL), dried, and evaporated. Purification by rapid silica filtration (1:19 MeOH:DCM) afforded maleimide **16** as a pale yellow powder (108 mg, 27%), identical to the material described above.

*1-(4-(((5-((3aR,4R,6aS)-2-Oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanoyl)oxy)amino)phenyl-1,2,3,4,5,6-$^{13}C_6$)-1H-pyrrole-2,5-dione (N-4-(D-biotinylamino-(1,2,3,4,5,6-$^{13}C_6$)phenyl)maleimide) (**16***).* Method B was applied to **14*** (50 mg, 0.24 mmol), D-(+)-biotin (**3**) (60 mg, 0.25 mmol), TCTU (252 mg, 0.710 mmol), and Hünig's base (0.16 mL, 0.71 mmol) in anhydrous DMF (2 mL). Purification as above gave **16*** as a pale yellow powder (25 mg, 24%). ^{1}H NMR (500 MHz, d_6-DMSO): δ 10.03 (s, 1H, NH), 7.80−7.87 and 7.47−7.54 (dm, J_{CH} = 165 Hz, 2H, H2‴/6‴), 7.34−7.42 and 7.03−7.11 (dm, J_{CH} = 155 Hz, 2H, H3‴/5‴), 7.16 (s, 2H, H3/4), 6.43 (s, 1H, NH), 6.35 (s, 1H, NH), 4.30 (dd, J_1 = 7.5 Hz, J_2 = 5.3 Hz, 1H, CH, H6a′), 4.12−4.16 (m, 1H, CH, H3a′), 3.10−3.15 (m, 1H, CH, H4′), 2.83 (dd, 1H, J_1 = 12.5 Hz, J_2 = 5.1 Hz, H6′), 2.58 (d, 1H, J = 12.5 Hz, H6′), 2.33 (t, 2H, J = 7.5 Hz, CH_2, H2″), 1.30−1.70 (m, 6H, 3 × CH_2, H3″/4″/5″). ^{13}C NMR (150 MHz, d_6-DMSO): δ 171.4 (C1″), 170.1 (C2/5), 162.7 (C2′), 138.8 (dt, J_1 = 62.5 Hz, J_2 = 9.6 Hz, C1′), 134.6 (C3/4), 126.8−127.9 (m, C3‴/5‴), 125.5−126.5 (m, C4‴), 118.6−119.6 (m, C2‴/6‴), 61.1 (C6a′), 59.2 (C3a′), 55.4 (C4′), 36.2 (C2″), 28.2 (C4″), 28.1 (C5″), 25.1 (C3″). HRMS (ESI+) m/z: calcd. for $C_{14}{}^{13}C_6H_{22}N_4O_4SNa^+$ $[M + Na]^+$ 443.1455, found 443.1465. NMR assignments were made with the assistance of COSY, HSQC, and HMBC experiments.

*5-((3aS,4S,6aR)-2-Oxohexahydro-1H-thieno[3,4-d]-imidazol-4-yl)pentanamide (**18**) (D-Biotinanilide).* The procedure for (**18**) was adapted from Feng et al.[43] A suspension of D-(+)-biotin (**3**) (54 mg, 0.22 mmol) and HCTU (87.5 mg, 0.212 mmol) in anhydrous DMF (1 mL) was stirred for 20 min, followed by addition of NEt_3 (0.20 mL, 1.4 mmol), forming a yellow solution. Aniline (**17**) (0.020 mL, 0.14 mmol) was added, and the solution was stirred at 80 °C overnight. The solvent was evaporated, and the brown residue was dissolved in 1:9 MeOH/DCM (5 mL). Solid $NaHCO_3$ (120 mg, 1.43 mmol) was added, and the suspension was stirred for 30 min, then filtered through a plug of silica. The filtrate was evaporated and the residue was purified by rapid silica filtration. Elution with 1:19 MeOH/DCM afforded benzamide **18** as a pale-beige powder (35 mg, 79%). IR (ATR) cm^{-1}: 3300 (NH), 1696 (C═O), 1659 (C═O). ^{1}H NMR (d_6-DMSO, 400 MHz) δ 9.87 (s, 1H, NH), 7.58 (d, J = 7.6 Hz, 2H, ArH, H3″/5″), 7.28 (dd [app. t], J_1 = J_2 = 7.5 Hz, 2H, ArH, H2″/6″), 7.01 (t, J = 7.4 Hz, H4″), 6.43 (s, 1H, NH), 6.35 (s, 1H, NH), 4.28−4.32 (m, 1H, CH, H6a), 4.12−4.16 (m, 1H, CH, H3a), 3.10−3.14 (m, 1H, CH_2, H4), 2.82 (dd, J_1 = 5.1 Hz, J_2 = 12.4 Hz, 1H, H6), 2.58 (d, J = 12.4 Hz, 1H, H6), 2.30 (t, J = 7.5 Hz, 2H, CH_2, H2′), 1.33−1.69 (m, 6H, 3 × CH_2, H3′/4′/5′). ^{13}C NMR (d_6-DMSO, 100 MHz): δ 171.6 (C1′), 163.1 (C2), 139.8 (C1″), 129.1 (C3″/5″), 123.4 (C4″), 119.5 (C2″/6″), 61.5 (C3a), 59.7 (C6a), 55.8 (C4), 36.7 (C6), 28.67 (C4′), 28.53 (C5′), 25.6 (C3′). HRMS (ESI−) m/z: calcd. for $C_{16}H_{20}N_3O_2S^-$ $[M-H]^-$ 318.1276; found 318.1281. NMR assignments were made with the assistance of COSY, HSQC, and HMBC experiments.

*N-(4-Nitrophenyl)-5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide (**19**) (N-biotinyl-4-nitroaniline).* The procedure was adapted from Quideau et al.[31] D-(+)-Biotin (**3**) (500 mg, 2.05 mmol) was suspended in $SOCl_2$ (10 mL, 140 mmol) under moisture guard and stirred until complete dissolution to give an orange solution. The excess thionyl chloride was evaporated under a stream of N_2, after which *p*-nitroaniline (**20**) (190 mg, 1.37 mmol) and pyridine (3.0 mL, 37 mmol) were added to form a brown suspension. Anhydrous DMF (1 mL) was added, and the resulting brown solution was stirred at room temperature for 4 days. The solvent was evaporated, and the residue was dissolved in 1:9 MeOH:DCM (5 mL) and stirred with solid $NaHCO_3$ (1.17 g, 14.0 mmol) for 30 min. The resulting suspension was filtered through a plug of silica gel. The filtrate was evaporated, and the residue was subjected to rapid silica filtration. Elution with DCM afforded *p*-nitroaniline **20** (63 mg, 33% recovery). Further elution with 1:19 MeOH:DCM afforded amide **19** as an orange powder (145 mg, 29%). IR (ATR) cm^{-1}: 3220 (NH), 1696 (C═O unresolved). ^{1}H NMR (d_6-DMSO, 400 MHz) δ 10.50 (s, 1H, NH), 8.21 (d, J = 9.2 Hz, 2H, ArH, H3″/5″), 7.28 (d, J = 9.2 Hz, ArH, H2″/6″), 6.43 (s, 1H, NH), 6.36 (s, 1H, NH), 4.28−4.32 (m, 1H, CH, H6a), 4.12−4.16 (m, 1H, CH, H3a), 3.10−3.14 (m, 1H, CH, H4), 2.82 (m, 1H, H6), 2.58 (d, J = 12.4 Hz, 1H, H6), 2.39 (t, J = 7.5 Hz, 2H, CH_2, H2′), 1.33−1.69 (m, 6H, 3 × CH_2, H3′/4′/5′). ^{13}C NMR (d_6-DMSO, 100 MHz) δ 172.7 (C1′), 163.2 (C2), 146.0 (C4″), 142.5 (C3′′/5′′), 125.5 (C1′′), 119.1 (C2′′/6′′), 61.5 (C6a), 59.7 (C3a), 55.9 (C4), 36.9 (C6), 28.66 (C4′), 28.58 (C5′), 25.3 (C3′). HRMS (ESI+) m/z: calcd. for $C_{16}H_{21}N_4O_4S^+$ $[M + H]^+$ 365.1284; found 365.1295. NMR assignments were made with the assistance of COSY, HSQC, and HMBC experiments.

Acet-$^{13}C_6$-anilide. The procedure was adapted from Lawrie.[32] Ac_2O (0.40 mL, 4.2 mmol) was added to a solution of $^{13}C_6$-aniline (250 mg, 2.52 mmol) in dry DCM (15 mL), and stirred for 1 h. The solution was washed with $NaHCO_3$ (3 × 10 mL), and the aqueous layer was back-extracted with EtOAc (2 × 10 mL). The combined organic phase was dried and evaporated to afford the title compound as beige crystals (299 mg, 84%). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.49 (m [app. dq], J_{CH} = 160 Hz, 2H, H2/6), 7.31 (m [app. dquint.] J_{CH} = 160 Hz, 2H, H3/5), 7.09 (m [app. dquint.], J_{CH} = 160 Hz, 1H, H4), 2.18 (s, 3H, CH_3), NH obscured. ^{13}C NMR ($CDCl_3$, 125 MHz): δ 168.6 (CO), 138.2 (dt, ^1J = 63.5 Hz, ^3J = 9.9 Hz, C1′), 129.3 (ddd [app. dt], $^1J_1$ = $^1J_2$ = 55.9 Hz, ^3J = 7.5 Hz, C2′/6′), 125.0 (ttd, ^1J = 55.8 Hz, ^2J = 2.5 Hz, ^3J = 9.9 Hz, C4′), 120.1 (m, C3′/5′), 25.0 (d, J = 2.5 Hz, CH_3). This compound has been synthesized previously,[32] but no analytical data were reported.

p-Nitroacet-$^{13}C_6$-anilide. Adapted from Lawrie.[32] $NaNO_3$ (260 mg, 3.08 mmol) was added to a stirred solution of acet-$^{13}C_6$-anilide (290 mg, 2.05 mmol) in conc. H_2SO_4 (5 mL) under a moisture guard at 0 °C. The reaction was stirred for 4 h, during which time a pale orange color developed. The solution was poured out onto ice/water (200 mL), and the precipitate was filtered and air-dried to afford the title compounds as a pale yellow powder (222 mg, 59%). ^{1}H NMR (500 MHz, d_6-DMSO) δ 10.55 (s, 1H, NH), 7.62−8.40 (m, 4H, ArH), 2.11 (1H, CH_3, s). ^{13}C NMR (125 MHz, d_6-DMSO) δ 168.6 (s, CO), 145.9 (dt, ^1J = 61.2 Hz, ^3J = 9.0 Hz, C4′), 142.4 (dt, ^1J = 67.0 Hz, ^3J = 9.0 Hz, C3′/5′), 125.4 (m, C2′/6′), 119.0 (m, C1′), 24.7 (d, J = 2.9 Hz, CH_3). HRMS (ESI+) m/z: calcd. for $^{12}C_2{}^{13}C_6H_9N_2O_3^+$ $[M + H]^+$ 187.0809, found 187.0814. This compound has been synthesized previously,[32] but no analytical data were reported.

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*$^{13}C_6$-p-Nitroaniline (**20***).* The procedure for the synthesis of **20*** was adapted from Lawrie.[32] A stirred suspension of *p*-nitroacet-$^{13}C_6$-anilide (212 mg, 1.17 mmol) in 35% aq. NaOH (10 mL) was heated at 50 °C for 12 h. After cooling, the aqueous solution was extracted with EtOAc (3 × 10 mL). The organic extract was dried and evaporated to afford **20*** as yellow crystals (160 mg, 97%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.87−8.26 (m, 2H, H3/5), 6.43−6.81 (m, 2H, H2/6), 4.31 (s, 2H, NH_2). ^{13}C NMR (125 MHz, $CDCl_3$): δ 152.6 (td, ^1J = 60.8 Hz, ^3J = 8.5 Hz, C1), 139.3 (br td, ^1J = 67.9 Hz, ^3J = 8.7 Hz, C4), 125.9−127.0 (m, C3/5), 113.0−114.0 (m, C2/6). HRMS (ESI+) *m*/*z*: calcd. for $^{12}C^{13}C_6H_{10}N_2O_3^+$ [M+H +MeOH]$^+$ 177.0966, found 177.0966. This compound has been synthesized previously,[32] but no analytical data were reported.

*N-(4-Aminophenyl)-5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide (**21**).* A suspension of 10% Pd/C (145 mg) in a solution of **19** (140 mg, 0.384 mmol) in anhydrous DMF (6 mL) was stirred under a balloon of H_2 overnight, after which time TLC (1:9 MeOH:DCM) indicated the reaction was complete. The mixture was diluted with MeOH (20 mL) and filtered through Celite. The solvent was evaporated to afford the slightly impure product **21** as an orange powder (115 mg, 90%). IR (ATR) cm^{-1}: 3243 (NH), 1690 (C═O), 1649 (C═O). ^{1}H NMR (d_6-DMSO, 400 MHz): δ 9.41 (s, 1H, NH), 7.19 (d, *J* = 8.7 Hz, ArH, H2″/6″), 6.47 (d, *J* = 8.7 Hz, 2H, ArH, H3″/5″), 6.43 (s, 1H, NH), 6.36 (s, 1H, NH), 4.28−4.32 (m, 1H, CH, H6a), 4.12−4.16 (m, 1H, H3a), 3.10−3.14 (m, 1H, H4), 2.82 (m, 1H, H6), 2.58 (d, *J* = 12.4 Hz, 1H, H6), 2.21 (t, *J* = 7.5 Hz, 2H, H2′), 1.33−1.69 (m, 6H, H3′/4′/5′). ^{13}C NMR (d_6-DMSO, 100 MHz): δ 170.6 (C1′), 163.2 (C2), 145.0 (C1″), 129.0 (C4″), 121.4 (C2″/6″), 114.2 (C3″/5″), 61.5 (C6a), 59.7 (C3a), 55.9 (C4), 36.9 (C6), 28.74 (C4′), 28.56 (C5′), 25.8 (C3′). HRMS (ESI−) *m*/*z*: calcd. for $C_{16}H_{21}N_4O_2S^-$ [M−H]$^-$ 333.1385; found 333.1377.

Animal Procedures. Mouse muscle samples were obtained from either adult male dystrophic mdx (C57Bl/10ScSnmdx/mdx) or non-dystrophic control C57Bl/10ScSn (C57) mice (the parental strain for mdx). All mice were obtained from the Animal Resource Centre, Murdoch, Western Australia. They were maintained at the University of Western Australia on a 12 h light/dark cycle, under standard conditions, with free access to food and drinking water. All animal experiments were conducted in strict accordance with the guidelines of the National Health and Medical Research Council Code of practice for the care and use of animals for scientific purposes (2004), and the Animal Welfare act of Western Australia (2002), and were approved by the Animal Ethics committee at the University of Western Australia.

As part of a broader study on oxidative stress in muscular dystrophy, dog muscle samples were obtained from dystrophic male golden retriever dogs or healthy controls, aged approximately 8 months. These dogs were handled and housed in the Boisbonne Center for Gene Therapy (ONIRIS, Atlantic Gene Therapies, Nantes, France). The Institutional Animal Care and Use Committee of the Region des Pays de la Loire (University of Angers, France) approved all the experimental protocols. Skeletal muscle samples (biceps femoris) were obtained after the dogs were sacrificed, performed by intravenous injection of pentobarbital sodium (Dolethal, Vetoquinol). Muscle samples were placed into sterile microtubes, frozen in liquid nitrogen, and subsequently stored at −80 °C until analysis.

Labeling of Lysozyme Standards. A 2 mg/mL solution of purified lysozyme was prepared in SDS/Tris buffer (0.5% SDS, 0.5 M Tris, pH 7.0). A lysozyme aliquot (200 μg) was reduced by adding 50 mM tris(2-carboxyethyl)phosphine (TCEP; pH 7.0) (4.8 μL). The sample was vortexed and incubated for 2 h at room temperature. More SDS/Tris buffer was added to dilute the reduced lysozyme solution to 100 μg/mL. DMSO solutions of ICMATs (5 mM) (**7**, **7***, **16**, and **16***) were stored at −20 °C. Aliquots of reduced lysozyme solution (400 μL, containing 40 μg lysozyme) were alkylated by the addition of 16 μL of 5 mM ICMAT solution (i.e., each ICMAT was added to a separate aliquot of reduced lysozyme at a ratio of 2 nmol ICMAT/μg lysozyme), followed by vigorous vortexing at room temperature for 1 h. Standards of different proportions of **7**:**7*** or **16**:**16*** labeled lysozyme (0:100, 20:80, 35:65, 50:50, 65:35, 80:20, and 100:0 ratios) were prepared such that each standard contained 8 μg of lysozyme. To remove excess tags and SDS, 1 mL of acetone, precooled to −20 °C, was added to each standard, briefly vortexed and incubated at −20 °C overnight. The samples were then centrifuged at 10 000 *g* for 5 min at 4 °C, and the supernatant was removed. The protein pellets were redissolved in of 100 mM pH 7.0 Tris (200 μL) and were digested at 37 °C for 16 h with trypsin (Promega), at 1:25 protease:protein (w/w) ratio. Each sample was then split into 4 μg protein aliquots and desiccated by vacuum centrifugation.

Labeling of Muscle Samples. The labeling technique used was adapted from the fluorescent method for quantifying protein thiol oxidation,[44,45] involving the sequential labeling of reduced and oxidized protein thiol groups, using ICMATs (either the **7**/**7*** pair or the **16**/**16*** pair). Frozen muscle (∼10 mg) was crushed under liquid nitrogen, and suspended in ice-cold 20% (w/v) trichloroacetic acid (TCA)/acetone (500 μL), then homogenized (Ultra-Turrax T25; Rose Scientific) on maximum for 15 s to produce an even suspension, which was incubated at −20 °C for at least 1 h. An aliquot of the suspension (50 μL) was diluted with acetone precooled to −20 °C (1 mL), then centrifuged at 10,000 *g* for 5 min at 4 °C. The supernatant was discarded and the protein pellet was dissolved in 0.5% pH 7.3 SDS/Tris buffer (300 μL). Native protein thiols were labeled with either ICMAT-1 (**7**) or ICMAT-3 (**16**) by adding a 5 mM DMSO solution (30 μL; final concentration, 0.46 mM) followed by incubation for 30 min at room temperature. Excess **7** or **16** ICMAT was consumed by the addition of 50 mM aqueous cysteine (6.5 μL; final concentration ∼1 mM cysteine). Oxidized thiols were reduced with 3.7 mM TCEP in 0.5% pH 7.3 SDS/Tris buffer, then labeled with 5 mM **7*** or **16*** in DMSO (respectively, depending on which ICMAT pair was being used in the experiment) to a final ICMAT concentration of 0.046 mM. Labeled protein was recovered by acetone precipitation (6:1 v/v) and centrifugation at 3000 *g* for 15 min. Protein was digested by addition of a solution of trypsin (20 μg) in 100 mM TRIS pH 7.0 (520 μL) and incubation at 37 °C for 16 h. Digested proteins (peptides) were purified using the cation exchange and affinity purification columns provided with the SCIEX Cleavable ICAT kit, as per manufacturers specifications (Sciex). Purified peptide fractions were desiccated using vacuum centrifugation.

StrataX Polymeric Reversed Phase Desalting. Each StrataX column was preconditioned with MeOH (2 mL) then

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MeCN (2 mL), followed by 80% MeCN/H_2O/0.1% formic acid (0.5 mL) then water (1 mL). Peptide sample were reconstituted in water (200 μL), then loaded onto the preconditioned StrataX column, washed with water (2 mL), and eluted with 80% MeCN/H_2O/0.1% formic acid (0.5 mL). All samples were then desiccated by vacuum centrifugation and stored at −80 °C until analysis by mass spectrometry.

Matrix-Assisted Laser Desorption/Ionization (MALDI-TOF/TOF) Mass Spectrometry. Labeled peptide samples derived from lysozyme were reconstituted in 80% MeCN/H_2O/0.05% TFA (5 μL). Peptide solutions (0.6 μL) were combined with matrix solution (0.6 μL 5 mg/mL α-cyano-4-hydroxysuccinamic acid, 10 mM ammonium citrate, 80% MeCN/H_2O/0.1% TFA) on a MALDI-TOF/TOF plate, and allowed to air-dry. Analysis was performed with a 5800 MALDI-TOF/TOF Mass Spectrometer (AB Sciex). Parent mass peaks (mass range m/z 800−4000 from combined MS and MS/MS spectra) were submitted to the MASCOT database for confirmation of labeled lysozyme peptides, using the following search conditions: Swissprot database, all mammalian species, trypsin digest with allowance for up to one missed cleavage per peptide, no fixed modifications, variable modification of oxidation on methionine residues and alkylation of cysteine residues with ICMATs, MS tolerance of 0.4 Da, MS/MS tolerance of 0.4 Da. Areas under the peaks corresponding to the labeled lysozyme peptide CELAAAMK were obtained from the TOF/TOF Explorer Series software (v 4.1.0, AB Sciex) to quantify the experimental ratio of the isotopic ICMATS.

HPLC−MS/MS. Each labeled peptide sample was reconstituted in solvent A (2% MeCN/0.1% formic acid) at approximately 0.1 μg/μL, and a volume of 20 μL was injected into a Shimadzu Prominence nano HPLC system coupled to a 5600 TripleTOF (Sciex) mass spectrometer. Separation was achieved on an Agilent Zorbax 300SB-C18, 3.5 μm column eluted with a linear gradient of H_2O/MeCN/0.1% formic acid (v/v) at 40 °C. The HPLC mobile phase was prepared by combining 0.1% formic acid and 2% MeCN in H_2O (A) and 0.1% formic acid and 2% H_2O in MeCN (B). Gradient elution was performed with 2% B for 3 min, increased to 40% B at 15 min, then ramped to 98% B by 16 min and held for 2 min before returning to 2% B within 1 min. MS spectra were acquired using electrospray ionization in mode, by information-dependent acquisition (IDA), whereby only the most intense 20 MS peaks between 400 and 1250 m/z were selected for MS/MS scans. Mass tolerance was 50 mDa. A calibration with trypsin-digested bovine serum albumen (BSA) was conducted before a batch of samples was run.

For labeled lysozyme standards, areas under the peaks of the extracted ion chromatograms corresponding to the labeled lysozyme peptide CELAAAMK were obtained from the Skyline software (AB Sciex, v 4.1.0) to quantify the experimental ratio of the isotopic ICMATs. Sample preparation variation was accounted for with a correction factor calculated from the 50:50 light:heavy sample. Quantification of muscle-derived peptides was performed in the same manner, but without the correction factor.

MS/MS Proteomic Analysis. MS/MS data were imported into the database search engine Mascot (v 2.4.1, www.matrixscience.com) for identification of peptides and proteins, using the following search conditions: Swissprot database, all mammalian species, trypsin digest with allowance for up to one missed cleavage per peptide, no fixed modifications, variable oxidation of methionine residues, and alkylation of cysteine residues (by the relevant ICMAT). Other search criteria included a MS tolerance of 0.2 Da nd MS/MS tolerance of 0.2 Da. Protein identification was determined on the basis of two or more identified peptides with ion scores exceeding the significance threshold.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.bioconjchem.1c00206.

^{1}H and ^{13}C NMR spectra of all new compounds (PDF)

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Notes

The authors declare no competing financial interest.

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