

N-Myristoyl transferase-mediated protein labelling *in vivo*

William P. Heal,^a Sasala R. Wickramasinghe,^a Robin J. Leatherbarrow,^{*ab} and Edward W. Tate^{*ab}

Received (in XXX, XXX) 1st January 2007, Accepted 1st January 2007

First published on the web 1st January 2007

DOI: 10.1039/b000000x

N-Myristoyl transferase-mediated labelling using a substrate modified with an azide or alkyne tag is described as an efficient and site-selective method for the introduction of a bioorthogonal tag at the N-terminus of a recombinant protein. The procedure may be performed *in vitro*, or in a single over-expression/tagging step *in vivo* in bacteria; tagged proteins may then be captured using Staudinger-Bertozzi or ‘click’ chemistry protocols to introduce a secondary label for downstream analysis. The straightforward synthesis of the chemical and molecular biological tools described should enable their use in a wide range of N-terminal labelling applications.

Introduction

Protein labelling using bioorthogonal ligation chemistry

Site-specific labelling of proteins with chemical or fluorescent probes is a prerequisite for many established and emerging techniques in chemical and cell biology.¹⁻³ Protein labelling technology has been revolutionised by the discovery of highly selective, water-compatible reactions that permit bioorthogonal ligation of a synthetic probe at a defined site that bears a small chemical tag such as an azide, alkyne or aldehyde.⁴ Bioorthogonal ligation chemistry has proven to be robust,⁵ and has been used in a remarkable range of applications for labelling proteins and other biomolecules both *in vitro* and *in vivo*.⁶ However, there remains a need for methods that allow the introduction of suitable tags into a protein in both an efficient and site-specific manner, particularly in live cells.

Two distinct approaches have been developed to address this problem, which enable introduction of tagged sites at either the DNA or protein level. Expanded codon usage, first pioneered by the Schultz group, enables incorporation of a tagged amino acid at an arbitrary number of specific sites encoded in the parent gene.^{7,8} Alternatively, the protein can be modified post-translationally to introduce a chemical modification bearing a bioorthogonal tag at a suitable consensus motif. A growing range of enzymes have been adapted for use in this approach, including farnesyl transferases,^{9,10} biotin ligase¹¹ and formylglycine-generating enzyme.¹² Despite the great potential of these techniques, their adoption for general labelling applications can be limited by the need for relatively complex synthetic and biochemical protocols.

We recently communicated¹³ a straightforward and practical transferase-based technique that permits site-specific *in vitro* and *in vivo* generation of N-terminal azide-tagged recombinant protein, and subsequent labelling with Staudinger-Bertozzi ligation chemistry.¹⁴ Here we report this work in full, and extend the scope to encompass transferase-mediated *in vivo* production of alkynyl-tagged proteins and subsequent bioorthogonal labelling with [3 + 2] azide-alkyne cycloaddition (‘click’ chemistry).⁴

Myristoyl-CoA:protein N-myristoyl transferase (NMT)

N-Myristoylation at an N-terminal glycine is a constitutive co-translational modification that occurs in all eukaryotic cells, and contributes to regulation of signalling and trafficking by modulating protein-membrane and protein-protein associations.¹⁵ Myristoyl-CoA:protein N-myristoyl transferase (NMT), the enzyme that catalyses myristoylation, has been characterised in a wide range of organisms, from yeast to humans.¹⁶⁻²¹ Comprehensive studies by Gordon *et al.* found that the specificity of *Saccharomyces cerevisiae* NMT (ScNMT) towards fatty acyl-coenzyme A (CoA) analogues is limited to acids that closely mimic myristoyl-CoA (**2**, Fig. 1), particularly with respect to chain length (~14 heteroatoms) and flexibility.²²⁻²⁴ Nevertheless, this early work suggested that ScNMT tolerates the introduction of some functionality in the acyl chain of the myristoyl-CoA substrate and recent reports have also described the use of modified myristate for non-radioactive metabolic labelling in mammalian cell lines.^{25,26} Encouraged by this work, we decided to investigate transfer of alkyne- and azide-modified myristate analogues by NMT as a means for introducing a bioorthogonal ligation tag at the N-terminus of proteins bearing an N-terminal myristoylation motif.

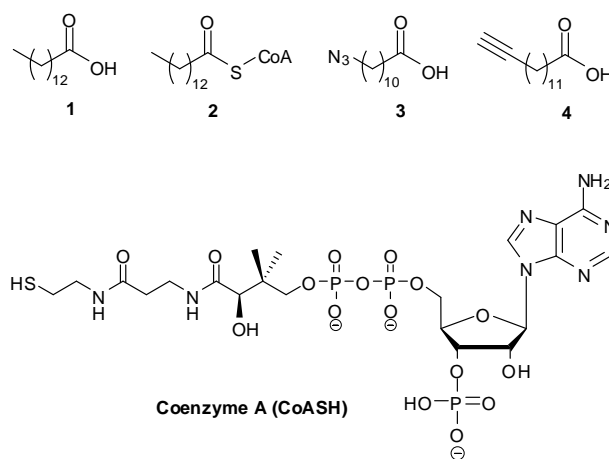


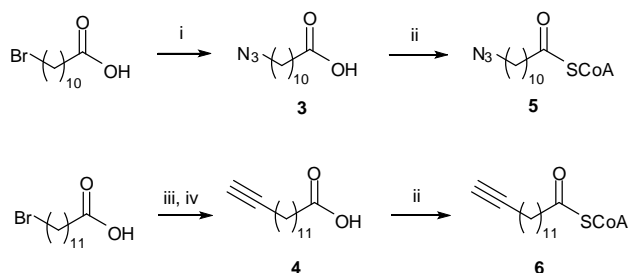
Fig. 1 Myristic acid **1**, myristoyl-CoA **2** and myristic acid analogues **3** and **4** designed and used in this study.

Results and Discussion

Design and synthesis of tagged substrates for NMT

A well-characterised and widely-used NMT cloned from *Candida albicans* (CaNMT)¹⁸ was selected as the azido- or alkynyl-myristate transfer agent. This enzyme exhibits excellent stability, activity and solubility, and may be over-expressed and purified without the need for an affinity tag.²⁷ Two myristic acid analogues were designed, incorporating an azide (**3**) or alkyne (**4**) at the terminal position (Fig. 1) so as to mimic the chain length and flexibility of the natural substrate as closely as possible. In 11-azido undecanoic acid **3**, three carbon atoms were replaced by the three nitrogen atoms of the azide, whilst in tetradec-13-ynoic acid **4**, the terminal single bond was replaced by a triple bond. It was hypothesised that these relatively minor modifications would not have a significant impact on the affinity of the acyl-CoA substrate for CaNMT.^{18,27} The long alkyl chain in **3** and **4** would also be expected to act as a flexible linker between protein and tag, potentially serving to improve the accessibility of the tag for subsequent modification.

3 and **4** were synthesised in one or two steps starting from commercially-available ω -bromo acids (Scheme 1). Simply reacting 11-bromo undecanoic acid with sodium azide in DMSO furnished **3** in excellent yield, whilst a sequence of bromine displacement from 12-bromo dodecanoic acid with lithium trimethylsilyl acetylide followed by base-mediated deprotection gave **4** in good overall yield. Each analogue was then converted to the corresponding acyl-CoA thioester by activation with 1,1'-carbonyldiimidazole (CDI) and reaction with CoASH (trisodium salt) under basic conditions.



Scheme 1 Synthesis of myristic acid analogues **3** and **4**, and their corresponding CoA thioester analogues **5** and **6**. Reagents and conditions: i) Na₃N, DMSO; ii) a) CDI, THF, b) CoASH, THF-NaHCO₃ (0.5 M); iii) TMS-Acetylene, ^tBuLi, THF-HMPA, -78 °C; iv) K₂CO₃, MeOH.

In vitro azido- and alkynyl-tagging of a peptide substrate

A series of initial experiments were performed to demonstrate that **5** and **6** are indeed substrates for CaNMT. A model peptide substrate H-GLYVSRLFNRLFQKK-NH₂ (**7**) bearing a canonical N-terminal myristoylation motif of general sequence GXXXX (X = any amino acid)²⁸⁻³⁰ was synthesised by standard Fmoc/tBu solid phase peptide synthesis (SPPS). **7** corresponds to the N-terminal region of *Plasmodium falciparum* ADP ribosylation factor 1 (PfARF1),^{16,31-33} which we have shown previously to be a substrate for CaNMT. The corresponding N-terminal azido-myristoylated (**8**) and alkynyl-myristoylated (**9**) analogues of **7** were synthesised by capping the resin with **3** or **4** respectively prior to cleavage.

Incubation of equimolar quantities of **7** and one of the two acyl-CoA substrates (**5** or **6**) in the presence of a catalytic quantity of CaNMT (0.25 mol %) in a suitable buffer for up to 2 hours was sufficient for complete transfer of the acyl group to the target peptide as determined by HPLC (Fig. 2). In each case **7** was rapidly consumed during the reaction. Interestingly, transfer from the alkyne-tagged myristoyl-CoA analogue **6** appears to be at least two-fold faster than from the azide-tagged substrate **5**, which may reflect a higher affinity for the selective myristoyl-CoA binding pocket. Both analogues are transferred at a rate comparable to that of the native substrate **2**.²⁷

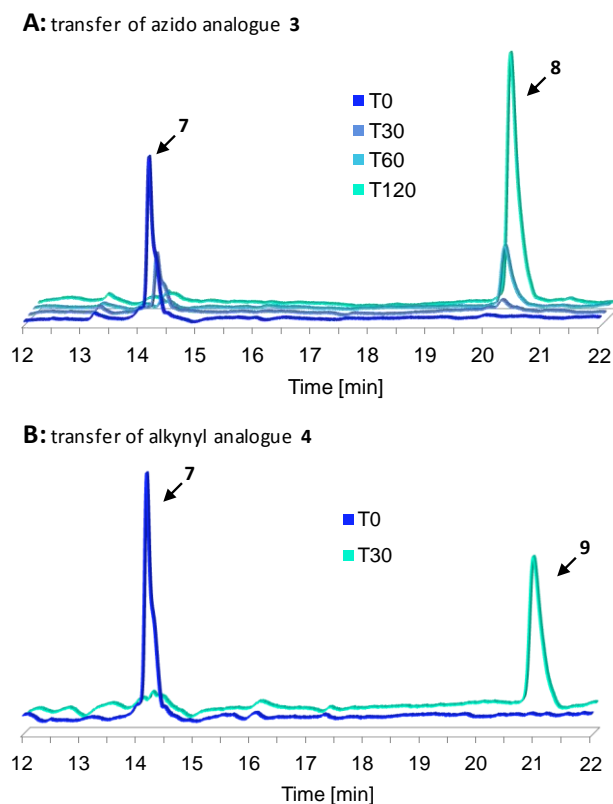


Fig. 2 HPLC traces showing *in vitro* transfer of A) **3** or B) **4** (B) via CoA ester **5**, or **6** to the N-terminus of peptide **7** (H-GLYVSRLFNRLFQKK-NH₂), over reaction times of T = 0 up to 120 min. Products were identified by reference to authentic samples of **8** and **9** prepared by chemical synthesis (see Experimental).

In vivo azido- and alkynyl-tagging of a recombinant protein

Having confirmed that CaNMT will transfer the tagged acyl group from acyl-CoA substrates **5** and **6** *in vitro*, we next sought to apply this technique for tagging and subsequent labelling of a recombinant protein.[†] Previous work has shown that *E. coli* engineered to co-express NMT and a substrate protein provides a practical system to study myristoylation *in vivo* in isolation from other cellular processes.^{34,35} Whilst bacteria are competent to convert myristic acid to the activated CoA thioester *via* acyl-CoA synthetase³⁶ they do not possess protein N-myristoyl transferases, which are an enzyme class exclusive to eukaryotes. Furthermore, in common with eukaryotes, bacteria express methionine aminopeptidase (MetAP),³⁷ which is required to strip the leader methionine

from a substrate protein to reveal the requisite N-terminal glycine. These properties make the bacterial co-expression system a useful tool for the production of N-myristoylated proteins.

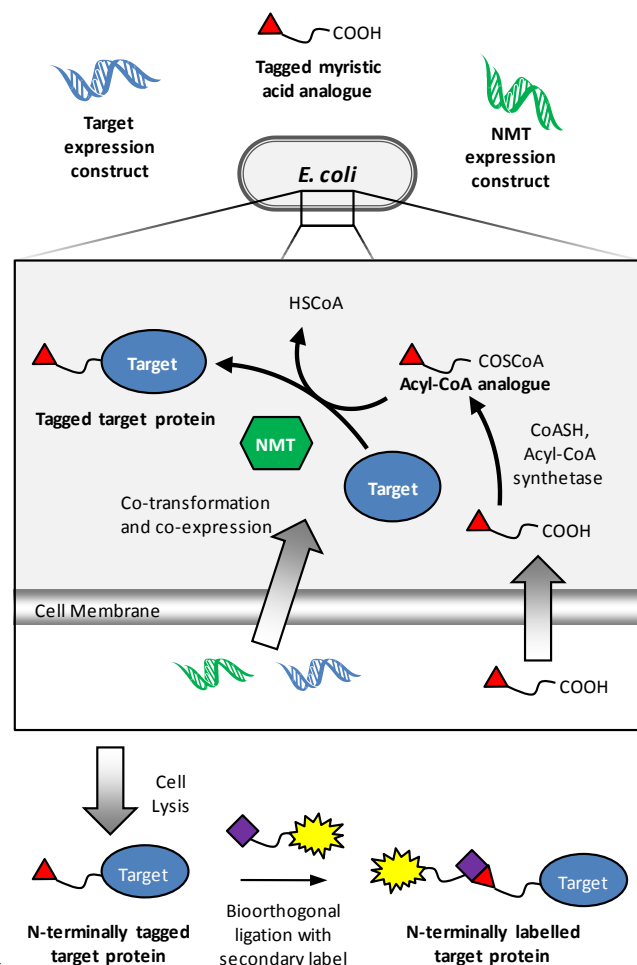


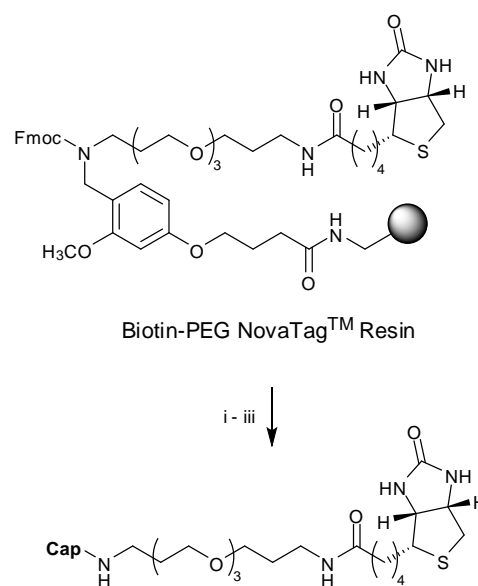
Fig. 3 Schematic overview of the *E. coli* co-expression system applied to azide and alkyne tagging of a protein *in vivo*.

In the co-expression approach bacteria are transformed with a pair of expression constructs containing the NMT and substrate sequences together with orthogonal antibiotic resistance genes; under double antibiotic selection the bacteria retain both plasmids. Simultaneous feeding with myristic acid and induction in the presence of isopropyl β -D-1-thiogalactopyranoside (IPTG) causes the bacteria to express both proteins, and myristic acid is transferred to the substrate inside the bacteria. We hypothesised that acids **3** and **4** could be used directly in such a co-expression system to enable the generation of tagged protein, as outlined in Fig. 3. The success of this approach would depend on both sufficient uptake of the myristic acid analogues and their acceptance as substrates by bacterial acyl-CoA synthetase. This methodology would offer two key advantages over the *in vitro* approach:

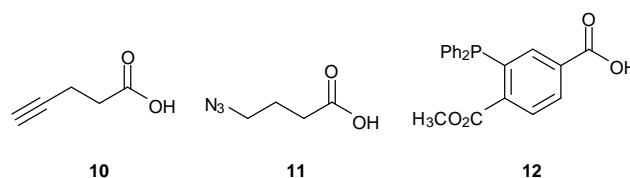
- It removes the requirement for synthesis of the acyl-CoA thioester, thus limiting the reagents required to cheap and readily available compounds.
- Expression and isolation of the target tagged protein can

be achieved in a single experiment, without the need for separate purification and isolation of either NMT or substrate.

A set of capture reagents were designed and synthesised to enable the detection of protein tagging *via* bioorthogonal ligation of a biotin label to either azide- or alkyne-tagged targets (Scheme 2). The syntheses described here are significantly simplified from those reported in previous studies, and can be carried out in as few as three steps, starting from commercially-available Biotin-PEG NovaTag™ resin. In each case, an acid bearing the appropriate capture group (pent-4-ynoic acid **10**, 4-azidobutyric acid **11** or 3-(diphenylphosphino)-4-(methoxycarbonyl) benzoic acid **12**) was preactivated with HATU and coupled in 4-fold excess relative to resin loading. Subsequent cleavage (95% aqueous TFA) and purification by RP-HPLC afforded the corresponding capture reagents bearing an azide (**13**), alkyne (**14**) or triarylphosphine (**15**) moiety in good overall yield.



- 13:** Cap = pent-5-ynoyl
14: Cap = 4-azidobutanoyl
15: Cap = 3-(diphenylphosphino)-4-(methoxycarbonyl) benzoyl



Scheme 2 Synthesis of capture reagents **13**, **14** and **15**. i) 20% piperidine/DMF; ii) acid **10**, **11** or **12**, HATU, DIPEA; iii) 95% TFA_(aq).

Expression constructs for CaNMT under ampicillin selection and PfARF1 under kanamycin selection were co-transformed into *E. coli* BL21 (DE3) competent cells, and induced for 4 hours at 37 °C in the presence of 1 mM IPTG together with 0.5 mM **3** or **4**. After cell lysis and centrifugation the fraction containing soluble protein was reacted with the appropriate capture reagent (see Experimental) and analysed by SDS-polyacrylamide gel

electrophoresis (SDS-PAGE). The PfARF1 construct bears a C-terminal His₆ tag, which enables verification by Western blot with α His₆-HRP conjugate (Fig. 4A), whilst biotinylated protein was visualised after blotting with NeutrAvidinTM-HRP conjugate (Fig. 4B–C). Negative control cells expressing only CaNMT (lanes 3, 4 and 8) or PfARF1 (lanes 7 and 8), cells fed with myristic acid rather than a tagged analogue (lane 5) and uncaptured lysates (lane 6) were run for comparison.

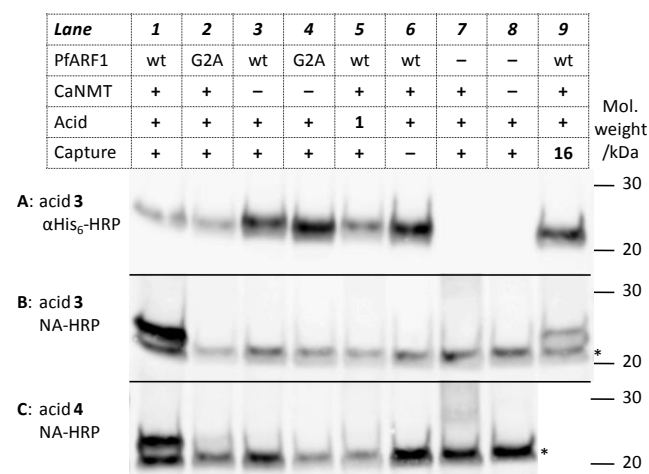


Fig. 4 *In vivo* transfer of an azide or alkyne tag to recombinant PfARF1. A: cells fed with azido acid **3** visualised by Western blot with anti-His₆-HRP conjugate; B: Cells fed with **3** and captured with reagent **13** (except lane 9, captured with reagent **15**), visualised with NeutrAvidinTM-HRP conjugate; C: Cells fed with **4** and captured with reagent **14** (except lane 9, experiment not performed), visualised with NeutrAvidinTM-HRP conjugate. A known endogenous biotinylated *E. coli* protein of ca. 22 kDa is observed in all samples in B and C (marked by *).³⁸

As shown in Fig. 4, transfer of both azido- and alkynyl-acids are observed only in the presence of CaNMT, and subsequent capture is highly specific for the tagged substrate. No significant capture of either myristoylated PfARF1 or endogenous *E. coli* proteins is observed under the conditions used. Furthermore, no labelling is observed in cells over-expressing CaNMT with a PfARF1[G2A] mutant protein bearing an alanine at the N-terminus (Fig. 4B and C, lanes 2 and 4), demonstrating that the present method is highly site-specific for an N-terminal Gly residue. The band observed below PfARF1 (Fig. 4B and C, lanes 1–9) is biotin carboxyl carrier protein (BCCP), a well-characterised endogenous biotinylated *E. coli* protein.^{38,41} Interestingly, the efficiency of capture by [3 + 2] cycloaddition (or ‘click’ chemistry) appears to be superior to that of capture with the Staudinger-Bertozzi reagent under the conditions used, an observation that corroborates a previous comparative study of bioorthogonal ligation approaches.⁵ [3 + 2] capture of azide tagged protein with **14** is also superior to that of alkyne tagged protein with **13** under identical conditions, an observation noted previously by Cravatt and co-workers.³⁹ This is likely due to the enhancement in rate in the presence of a large excess of activated alkyne in the case of capture using reagent **14**; in the case of capture with **13**, the rate is presumably limited by the relatively lower concentration of activated alkynyl-protein.

Conclusions

In summary, we have reported a novel method for *in vivo* co-translational site-specific introduction of an azide or alkyne chemical tag during expression of a recombinant protein. Subsequent elaboration *via* click chemistry has been used to introduce a biotin label for visualisation by gel electrophoresis, and in principle it should be possible to adapt this approach for the introduction of a wide range of labels. The canonical *N*-myristoylation motif GXXXS has been shown to direct N-terminal tagging to a single specific site. In addition to providing a convenient and potentially general method for N-terminal recombinant protein labelling, this system also provides a useful model system and proof-of-principle for *in vivo* ‘tagging-by-substrate’ studies aimed at elucidating the myristoylated proteome (or ‘myristome’) in cells in culture or in multicellular organisms.^{25,26} Post- and co-translational methods for protein labelling also present the potential for orthogonal multi-site protein labelling in combination with other enzymatic techniques. We envisage that the system presented here could be combined with related approaches reported for the post-translational introduction of tags,^{9,10,12} to enable site-specific labelling of a protein with a FRET pair, for example. Work is ongoing in our labs to extend the methodology presented here for the labelling of other recombinant proteins for biophysical and bioconjugate studies.

Experimental

Materials and Methods

All solvents were purchased from BDH and used without further purification. All reagents, unless otherwise stated, were purchased at the highest quality available from Sigma-Aldrich and used without further purification. NMR spectra were recorded in 5 mm tubes calibrated to the residual solvent peak stated, on Bruker AV-400 and AV-500 spectrometers. COSY and HMQC correlation spectra were used to aid assignment where necessary. Samples were characterised by MALDI-TOF (positive reflectron mode) mass spectrometry, recorded on a Micromass Autospec-Q spectrometer. Analytical RP-HPLC was carried out on a Gilson system (234 autoinjector, 322 series pumps, 155 UV/vis detector and controlled by the UnipointTM system interface) equipped with a Hichrom ACE 5 C18 250 × 4.6 mm analytical column. Semi-preparative RP-HPLC was carried out on a Gilson semi-preparative RP-HPLC system (Anachem Ltd.) equipped with type 306 pumps and a Gilson 151 UV/vis detector. The system was fitted with a Hichrom ACE 5 C18 250 × 21.2 mm semi-preparative column fitted with a SecurityGuardTM cartridge system, C18, 4 × 3.0 mm (Phenomenex). **Buffers:** Buffer A comprised 30 mM Tris, 2.5 mM DTT, 0.5 mM EGTA, pH 7.4; Buffer B comprised 30 mM Tris, 0.5 mM EGTA, 0.1% TRITON[®] X-100, pH 7.4; Buffer C comprised 50 mM Tris.HCl, pH 7.4, 200 mM sodium chloride and 0.1% TWEEN[®] 20; Buffer D comprised 50 mM Tris.HCl, pH 7.4, 200 mM sodium chloride and 0.1% TWEEN[®] 20, complete EDTA-free protease inhibitor cocktail (1 tablet per 15 mL, Roche Diagnostics); Buffer E comprised 1 × phosphate

buffered saline with 0.1% SDS and 0.1% TRITON® X-100.

Organic Synthesis

***ω*-Azido undecanoic acid (3):**²⁴ Sodium azide (1.37 g, 21.1 mmol) was dissolved in a solution of 11-bromoundecanoic acid (4.80 g, 17.5 mmol) in DMSO (200 mL). The clear colourless reaction mixture was stirred at room temperature overnight. The reaction mixture was diluted with water (80 mL) and to this was added HCl_(aq) (1 M, 30 mL) cautiously. Once the reaction mixture had cooled, the aqueous phase was extracted 3 × with EtOAc and the combined organic layers were washed 3 × with water and then brine before drying over MgSO₄. After filtration, all volatiles were removed under reduced pressure to yield the crude product as a pale yellow oil. Purification by flash column chromatography (diethyl ether–n-hexane, 1:1, R_f = 0.38) gave the product as a waxy solid (3.05 g, 77% yield). δ_H/ppm (400 MHz, CDCl₃) 1.24–1.43 (12H, m, (CH₂)₆), 1.55–1.72 (4H, m, CH₂CH₂CO₂H and CH₂CH₂N₃), 2.36 (2H, t, J = 7.5 Hz, CH₂CO₂H), 3.27 (2H, t, J = 7.0 Hz, CH₂N₃); δ_C/ppm (100 MHz, CDCl₃) 24.7, 26.7, 28.9, 29.0, 29.1, 29.2, 29.3, 29.4, 34.1, 51.5, 180.2 (C=O); m/z (MALDI-TOF –), 226 ([M – H][–]).

Tetradec-13-ynoic acid (4): To a solution of TMS-acetylene (1.26 mL, 8.95 mmol) in dry THF (7.0 mL) under nitrogen and cooled to –78 °C was added a solution of n-BuLi in hexanes (4.30 mL, 2.5 M, 10.74 mmol). The reaction mixture was allowed to warm up to room temperature for a few seconds before cooling to –78 °C. To this mixture was added a solution of 12-bromododecanoic acid (1.00 g, 3.58 mmol) in dry THF (20 mL) and dry HMPA (13 mL) dropwise. The reaction was allowed to warm to room temperature and stir until complete. Upon completion (after 18 hours, followed by TLC) the reaction mixture was returned to –78 °C and quenched by the addition of saturated NH₄Cl_(aq). The aqueous layer was extracted with DCM (3 × 50 mL) and the combined organic layers were washed with brine (3 × 50 mL) before drying over MgSO₄ and concentration under reduced pressure. The product was purified by flash column chromatography (n-hexane–ethylacetate (1:1), R_f = 0.48). The TMS protected alkyne was taken up in MeOH (10 mL) and to this was added K₂CO₃ (192.2 mg). The reaction mixture was stirred at room temperature for 18 hours, after which time all volatiles were removed under reduced pressure and the residues partitioned between ethylacetate–HCl_(aq) (1:1, 60 mL). The aqueous phase was extracted with ethylacetate (3 × 30 mL) and the combined organic phases dried and concentrated as before. The product was isolated as clear colourless oil and required no further purification (199.3 mg, 25%). δ_H/ppm (400 MHz, CDCl₃) 1.24–1.45 (14H, m, (CH₂)₇), 1.54 (2H, qi, J = 7.4 Hz, CH₂CH₂CO₂H), 1.65 (2H, qi, J = 7.0 Hz, CH₂CH₂C≡CH), 1.96 (1H, t, J = 2.6 Hz, C≡CH), 2.20 (2H, dt, J₁ = 2.6 Hz, J₂ = 7.0 Hz, CH₂C≡CH), 2.37 (2H, t, J = 7.5 Hz, CH₂CO₂H), 9.80 (1H, bs, CO₂H); δ_C/ppm (100 MHz, CDCl₃) 18.4, 20.7, 24.7, 28.5, 28.8, 29.1, 29.1, 29.2, 29.4, 29.5, 34.0, 68.1 (C≡CH), 84.8 (C≡CH), 180.0 (CO₂H) m/z (ESI-TOF –), 223 ([M – H][–]); HRMS, found 223.1710 (C₁₄H₂₃O₂, [M – H][–], requires 223.1698).

***ω*-Azido undecanoic acid CoA thioester (5):**²⁴ To a stirring solution of *ω*-azido undecanoic acid **3** (15 mg, 64 μmol) in dry THF (1.0 mL) under nitrogen was added a solution of CDI (12 mg, 77 μmol) in DCM (1.0 mL). The reaction mixture was allowed to stir at room temperature for 30 minutes. After this, all volatiles were removed under reduced pressure. The resultant solids were taken up in dry THF (2.0 mL) and to this was added a solution of CoASH (50 mg, 64 μmol) in aqueous NaHCO₃ (0.5 M, 5.0 mL). The reaction was allowed to stir at room temperature for 4 hours under nitrogen. After this time the THF was removed under reduced pressure and the remaining aqueous phase transferred in equal volumes to two 12 mL centrifuge tubes. The addition of 20% perchloric acid resulted in the formation of an off white precipitate. The solids were pelleted by centrifugation and washed 3 × with acetone. Purification by preparative RP-HPLC by elution over a gradient of MeCN in 10 mM NH₄OAc pH 5.2 (0–5 min 0% MeCN, 5–10 min up to 50% MeCN, 10–15 min 50% MeCN). Retention time = 23.3 min, detection was at 258 nm. The product was isolated as a white amorphous solid (11.4 mg, 17% yield). δ_H/ppm (400 MHz, D₂O) 0.70 (3H, s, CH₃), 0.84 (3H, s, CH₃), 1.08–1.29 (12H, m, 6 × CH₂), 1.41–1.54 (4H, m, CH₂CH₂CH₂C(O)NH and CH₂CH₂N₃), 2.34 (2H, s, SCH₂), 2.50 (2H, t, J = 7.2 Hz, (CH₂)₂CH₂C(O)NH), 2.90 (2H, t, J = 5.9 Hz, C(O)CH₂CH₂NH), 3.19 (2H, t, J = 6.9, CH₂N₃), 3.25 (2H, bs, C(O)CH₂CH₂NH), 3.36 (2H, bs, SCH₂CH₂), 3.50 (1H, bs, ½ × CH₂(CH₃)₂), 3.77 (1H, bs, ½ × CH₂(CH₃)₂), 3.95 (1H, bs, C(O)CHOH), 4.16 (2H, bs, Rib-CH₂), 4.51 (1H, bs, Rib-CH), 4.61–4.82 (2H, m, 2 × Rib-CH), 6.11 (1H, bd, J = 4.2 Hz, CH(Pur)), 8.31 (1H, s, Ar-H), 8.57 (1H, s, Ar-H); m/z (MALDI-TOF –), 1040 ([M + (NH₄⁺)₃ – H][–]).

Tetradec-13-ynoic acid CoA thioester (6): Following the above procedure (except using **4** (11.2 mg, 0.05 mmol), CDI (9.7 mg, 0.06 mmol) and CoASH (39.3 mg, 0.05 mmol)) the product was isolated as a white amorphous white solid (7.6 mg, 19%). δ_H/ppm (400 MHz, D₂O) 0.69 (3H, s, CH₃), 0.84 (3H, s, CH₃), 1.01–1.23 (14H, m, 7 × CH₂), 1.31 (2H, qi, J = 7.0 Hz, CH₂CH₂C≡CH), 1.45 (2H, bs, CH₂CH₂CH₂C(O)NH), 2.00 (2H, bs, CH₂C≡CH), 2.09 (1H, s, C≡CH), 2.35 (2H, s, SCH₂), 2.44 (2H, bs, (CH₂)₂CH₂C(O)NH), 2.88 (2H, bs, C(O)CH₂CH₂NH), 3.22 (2H, bs, C(O)CH₂CH₂NH), 3.36 (2H, bs, SCH₂CH₂), 3.51 (1H, bs, ½ × CH₂(CH₃)₂), 3.80 (1H, bs, ½ × CH₂(CH₃)₂), 3.95 (1H, bs, C(O)CHOH), 4.19 (2H, bs, Rib-CH₂), 4.50 (1H, s, Rib-CH), 4.64–4.80 (2H, m, 2 × Rib-CH), 6.06 (1H, bd, J = 4.8 Hz, CH(Pur)), 8.28 (1H, s, Ar-H), 8.54 (1H, s, Ar-H); that this compound did not generate satisfactory mass spectral data under ES (positive or negative mode), FAB or MALDI ionisation techniques. However, its purity (>95%) and identity were confirmed by proton NMR spectroscopy (see above) and by analytical RP-HPLC over a gradient of MeCN in 10 mM NH₄OAc pH 5.2 (0–5 min 0% MeCN, 5–10 min up to 50% MeCN, 10–15 min 50% MeCN). Retention time = 15.3 min, detection was by UV absorbance at 258 nm.

Solid-phase synthesis

Peptide Synthesis (H₂N-GLYVSRLFNRFLQKK-OH, 7):²⁷ N-α-9-Fluorenylmethoxycarbonyl (Fmoc) protected amino

acids were obtained (Novabiochem) with the following side chain protecting groups: Arg(Pbf), Asn(Trt), Gln(Trt), Gly, Leu, Lys(Boc), Phe, Ser(^tBu), Tyr(^tBu) and Val were purchased from Novabiochem. *O*-(Benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HBTU) and Rink amide MBHA resin were purchased from Merck Biosciences. Peptide synthesis grade *N,N*-dimethylformamide (DMF) was purchased from Rathburn Chemicals and HPLC grade acetonitrile from Fischer. All other chemicals were purchased from Sigma-Aldrich Company Ltd. All reactions were carried out under an atmosphere of nitrogen. An Advanced ChemTech Apex 396 multiple peptide synthesiser (Advanced ChemTech Europe) was used for automated peptide synthesis. The peptides created in this study used Rink amide MBHA resin as the solid support (providing a C-terminal amide). Syntheses utilised 25 µmol resin per well and a standard Fmoc/^tBu peptide synthesis strategy with DMF as solvent. The resin was swelled in DMF for one hour before proceeding with the cyclical steps of Fmoc deprotection (20% v/v piperidine in DMF), 3 × 5 min, washing (DMF, 5 × 1 min) and backbone elongation (coupling) mediated by HBTU and DIPEA (*N,N'*-diisopropylethylamine) (45 min). The coupling reaction used a 5-fold excess of amino acid to resin, 1 equivalent of HBTU and 2 eq. DIPEA. The coupling stage was repeated twice for the attachment of the first amino acid to the resin. After elongation and final Fmoc deprotection, the resin was washed with 1 × 1 mL DMF, 3 × 1 mL DCM and 3 × 1 mL MeOH before being allowed to dry in a desiccator overnight. Manual deprotection was accomplished by the addition of 1 mL of 95% TFA, 2.5% H₂O and 2.5% triisopropyl silane (TIS) with thorough mixing by vortex. The supernatant was displaced through the frit into a 12 mL centrifuge tube and the beads washed in a similar manner with a further 0.5 mL TFA. The combined washings were treated with ~9.5 mL TBME (cooled to -20 °C) to precipitate the peptides. The solids were pelleted by centrifugation for 15 min at 4300 rpm at 4 °C and washed (with vortexing and centrifugation) repeatedly in TBME (three times in total). The pelleted solids were dried in a desiccator overnight. The deprotected peptides were purified by semi-preparative RP-HPLC over a gradient of MeCN in 10 mM NH₄OAc pH 5.2 (0–5 min 0% MeCN, 5–10 min up to 50% MeCN, 10–15 min 50% MeCN). Detection was by UV absorbance at 223 nm. The product (retention time 14.2 min) was obtained by lyophilisation as a white amorphous solid. *m/z* (MALDI-TOF +), 1870 ([M+H]⁺).

Azidomystoylated Peptide Synthesis (AzMyr-NH-GLYVSRLFNRFLFQKK-OH, 8): The peptide was synthesised according to the procedure for **7**, except for addition of activated acid after removal of the final Fmoc group. A solution of HBTU (500 µL, 0.5 M) in DMF was added to a solution of **3** (125 µmoles in DMF, 0.5 M) and DIPEA (1.5 mL, 0.5 M). The resultant mixture was vortexed thoroughly for 15 min before addition to the appropriate well of the peptide synthesiser. The peptide was then washed and cleaved and purified as detailed above. The product (retention time 20.2 min) was recovered by lyophilisation as a white amorphous solid. *m/z* (MALDI-TOF +), 2078 ([M+H]⁺).

Alkynylmystoylated Peptide Synthesis (YnMyr-NH-GLYVSRLFNRFLFQKK-OH, 9): The peptide was prepared as for **8** except using **4** (125 µmoles in DMF, 0.5 M). The product (retention time 20.9 min) was recovered by lyophilisation as a white amorphous solid. *m/z* (MALDI-TOF +), 2075 ([M+H]⁺).

***N*-(15-Oxo-19-(2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)-4,7,10-trioxa-14-azanonadecyl)pent-4-ynamide (Alkynyl capture reagent, 13):** Biotin-PEG NovaTagTM resin (0.48 mmol g⁻¹ loading, 52.1 mg, 25 µmol) was deprotected (Fmoc) by treatment with 20% v/v piperidine in DMF before addition of pent-4-ynoic acid **10** (12.3 mg, 125 µmol), HATU (47.5 mg, 125 µmol) and DIPEA (43.5 µL) in DMF (2 mL). The crude product was cleaved from the resin by treatment with 95% TFA and purified by semi-preparative RP-HPLC over a gradient of MeCN (0.1% TFA) in H₂O (0.1% TFA) (0–1 min 30% MeCN, 1–30 min up to 100% MeCN, 30–35 min 100% MeCN). Detection was at 220 nm. The product was obtained by lyophilisation as a white amorphous solid (6.3 mg, 48% yield). δ_{H} /ppm (500 MHz, CDCl₃) 1.49 (2H, qi, *J* = 7.8 Hz, biotin CH₂), 1.64–1.85 (8H, m, biotin CH₂ × 2 and NHCH₂CH₂CH₂O × 2), 2.02 (1H, t, *J* = 2.6 Hz, C≡CH), 2.22 (2H, t, *J* = 7.4 Hz, biotin NHC(O)CH₂), 2.42 (2H, t, *J* = 7.3 Hz, CH₂CH₂C≡CH), 2.55 (2H, dt, *J* = 7.3, 2.5 Hz, CH₂C≡CH), 2.77 (1H, d, *J* = 12.8 Hz, biotin ½ × SCH₂), 2.95 (1H, dd, *J* = 12.8, 5.0 Hz, biotin ½ × SCH₂), 3.18 (1H, dt, *J* = 7.3, 5.6 Hz, SCH), 3.39 (4H, qi, *J* = 6.1 Hz, 2 × PEG CH₂NH), 3.57–3.71 (12H, m, 6 × PEG CH₂), 4.36 (1H, dd, *J* = 7.5, 4.7 Hz, biotin SCHCH), 4.54 (1H, dd, *J* = 7.6, 4.9 Hz, biotin SCH₂CH), 5.14 (1H, bs, NH), 5.96 (1H, bs, NH), 6.57 (2H, m, 2 × PEG-NHCO); δ_{C} /ppm (125 MHz, CDCl₃) 15.0, 25.6, 28.1, 29.0, 35.3, 35.4, 36.0, 37.8, 40.6, 55.5, 60.1, 61.9, 69.2, 69.9, 70.0 (4 × CH₂), 70.2, 70.5 (2 × CH₂), 83.3, 163.4, 171.0, 173.0; *m/z* (ESI-TOF +), 549 ([M + Na]⁺), 527 ([M + H]⁺); HRMS, found 527.2921 (C₂₅H₄₃N₄O₆S, [M + H]⁺, requires 527.2903).

***N*-(18-Azido-15-oxo-4,7,10-trioxa-14-azaoctadecyl)-5-(2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanamide (Azido capture reagent, 14):** Synthesis and purification as **13**, except for addition of 4-azidobutanoic acid **11** (16.1 mg, 125 µmol). The product was obtained by lyophilisation as a white amorphous solid (6.3 mg, 45% yield). δ_{H} /ppm (500 MHz, CDCl₃) 1.46 (2H, q, *J* = 7.4 Hz, biotin CH₂), 1.62–1.83 (8H, m, biotin CH₂ × 2 and NHCH₂CH₂CH₂O × 2), 1.92 (2H, qi, *J* = 7.2 Hz, CH₂CH₂N₃), 2.20 (2H, t, *J* = 7.3 Hz, biotin NHC(O)CH₂), 2.26 (2H, t, *J* = 7.3 Hz, CH₂CH₂CH₂N₃), 2.74 (1H, d, *J* = 12.6 Hz, biotin ½ × SCH₂), 2.92 (1H, dd, *J* = 12.8, 5.0 Hz, biotin ½ × SCH₂), 3.15 (1H, dt, *J* = 7.3, 5.6 Hz, SCH), 3.39 (6H, m, 2 × PEG CH₂NH and CH₂N₃), 3.54–3.68 (12H, m, 6 × PEG CH₂), 4.33 (1H, dd, *J* = 7.5, 4.7 Hz, biotin SCHCH), 4.51 (1H, dd, *J* = 7.6, 4.9 Hz, biotin SCH₂CH), 5.15 (1H, bs, NH), 5.95 (1H, bs, NH), 6.54 (2H, m, 2 × PEG-NHCO); δ_{C} /ppm (125 MHz, CDCl₃) 24.9, 25.6, 28.1, 29.0, 33.2, 35.9, 35.9, 37.8, 38.0, 40.6, 51.0, 55.5, 60.2, 61.9, 69.9, 70.0 (3 × CH₂), 70.2, 70.4, 70.5, 163.5, 172.0, 173.0; *m/z* (MALDI-TOF +), 580 ([M + Na]⁺), 558 ([M + H]⁺); HRMS, found 558.3074 (C₂₄H₄₄N₇O₆S, [M + H]⁺, requires 558.3074).

Methyl 2-(diphenylphosphino)-4-(15-oxo-19-(2-

oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)-4,7,10-trioxa-14-azonanadecylcarbonyl)benzoate (Staudinger-Bertozzi Capture Reagent, **16):** Synthesis and purification as **13**, except for addition of 3-(diphenylphosphino)-4-(methoxycarbonyl) benzoic acid **12**⁴⁰ (16.1 mg, 125 μmol). The product was obtained by lyophilisation as a white amorphous solid (9.9 mg, 50% yield). δ_{H} /ppm (400 MHz, CDCl₃) 1.43 (2H, q, $J = 7.6$ Hz, biotin CH₂), 1.60–1.87 (8H, m, biotin CH₂ × 2 and NHCH₂CH₂CH₂O × 2), 2.19 (2H, dt, $J = 1.6, 7.1$ Hz, biotin NHC(O)CH₂), 2.74 (1H, d, $J = 12.9$ Hz, biotin ½ × SCH₂), 2.91 (1H, dd, $J = 4.9, 12.9$ Hz, biotin ½ × SCH₂), 3.15 (1H, dt, $J = 4.7, 7.4$ Hz, biotin SCH), 3.33 (2H, app. q, $J = 5.9$ Hz, CH₂NHC(O)CH₂), 3.41–3.66 (14H, m, 6 × PEG CH₂ and ArC(O)NHCH₂), 3.75 (3H, s, OCH₃), 4.33 (1H, dd, $J = 4.6, 7.5$ Hz, biotin SCH₂CH), 4.52 (1H, dd, $J = 4.9, 7.7$ Hz, biotin SCH₂CH), 5.53 (1H, bs, NH), 6.24 (1H, bs, NH), 6.55 (1H, bs, NH), 7.01 (1H, bt, $J = 4.1$ Hz, NH), 7.26–7.38 (10H, m, PPh₂), 7.42 (1H, dd, $J = 1.5, 3.7$ Hz, Ar-H), 7.79 (1H, dd, $J = 1.5, 8.0$ Hz, Ar-H), 8.09 (1H, dd, $J = 3.7, 8.0$, Ar-H); δ_{C} /ppm (100 MHz, CDCl₃) 8.5, 25.5, 28.1, 28.3, 31.5, 35.7, 39.7, 45.8, 52.3, 55.6, 60.4, 61.9, 68.8, 69.7, 69.8, 70.0, 70.1, 70.3, 126.8, 128.6, 128.7, 128.9, 129.0, 130.8, 133.0, 133.8, 134.0, 136.5, 136.7, 137.2, 141.1, 141.4, 162.7, 164.5, 166.6, 166.8, 173.9; δ_{P} /ppm (162 MHz, CDCl₃) –3.77 (1P, s, PPh₂), 32.67 (1P, s, P(O)Ph₂), oxidised to unoxidised ~1:50 by peak integration; m/z (MALDI-TOF +), 815 ([M + Na]⁺), 793 ([M + H]⁺), 245 (biotin fragment); HRMS, found 793.3403 (C₄₁H₅₄N₄O₈SP, [M + H]⁺, requires 793.3400). IMPORTANT NOTE: DMSO stocks of **16** were found to be 90% oxidised to the corresponding phosphine oxide (by analytical RP-HPLC) after 1 month of constant use. To ensure the quality of **16** used in capture experiments, once a DMSO stock had been made up it was thoroughly de-gassed with helium, aliquotted into 20 μL portions and stored at –20 °C under N_{2(g)}.

HPLC transfer assay

Stock solutions containing azidomristoyl–CoA **4** (20 mM in 10 mM NaOAc pH 5.0–EtOH, 1:1), peptide **5** (20 mM in H₂O) and CaNMT (50 μM in buffer A, 20% glycerol) were prepared. Transfer reactions were carried out as follows: To buffer A (115 μL) was added CaNMT (1 μL), azidomristoyl–CoA **4** (5 μL) and peptide (5 μL), mixed thoroughly and the reactions incubated at 37 °C. Samples were taken at 0 h, 1 h, and 18 h. Sampling involved taking a 25 μL aliquot of the reaction mixture and quenching this into 25 μL of methanol, the resultant solution was analysed by HPLC over a gradient of MeCN (0.1% TFA) in H₂O (0.1% TFA) (0–2 min 2% MeCN, 2–25 min up to 100% MeCN, 25–35 min 100% MeCN). Detection was at 223 nm.

Protein expression and *in vivo* tagging

Recombinant CaNMT and PfARF1: CaNMT was cloned into pET11c (ampicillin resistance), expressed in *E. coli* and purified as described.²⁷ PfARF1 wild-type and G2A mutant proteins were cloned into pET28a (kanamycin resistance), expressed in *E. coli* and purified as reported previously.²⁷

Preparation of soluble cell extracts: BL21 (DE3) competent

cells (Stratagene) were co-transformed with pET11c–CaNMT and pET28a–PfARF1 (wild type) or PfARF1 (G2A mutant). Single transformations of each plasmid were performed as negative controls. A single colony of transformed cells was used to inoculate fresh Luria-Bertani media supplemented with the relevant selection antibiotics. Cells were incubated at 37 °C with shaking. At mid-log phase, protein expression was induced with 1 mM final IPTG, and the culture medium was supplemented with the appropriate acid (stock in DMSO) to a final concentration of 500 μM. Cells were induced for 4 hours at 37 °C with shaking. Cells were harvested by centrifugation and washed 3 times with buffer D. Cells were lysed by adding BugBuster[®] reagent (Novagen) supplemented with benzonase (Sigma-Aldrich). Soluble extracts were separated by centrifugation (17,000 × *g*, 30 m, 4 °C). Total protein content was determined by the Bradford method using bovine serum albumin (BSA) as a standard.

***In vivo* Assay:** To an aliquot of cell lysate containing 40 μg protein was added 0.3 μL capture reagent stock solution (10 mM in DMSO, 50 μM final), 6 μL TCEP stock solution (10 mM in H₂O, 1 mM final), 0.6 μL triazolyl ligand (10 mM in DMSO, 100 μM final), 6 μL CuSO₄ stock (10 mM in H₂O, 1 mM final) and the volume adjusted to 60 μL with buffer E if required. The resultant reaction mixtures were adigated at room temperature in a carousel for 1 hour. Two 15 μL aliquots were taken for SDS-PAGE (for detection of the His₆ tag and of biotin) with the same volume left for repeat analysis if required.

Gel-based analysis

SDS PAGE: Proteins were separated by SDS-PAGE using 1 mm Bis-Tris gels (4% stacking gel, 12% resolving gel) in NuPAGE[®] MES SDS running buffer (Invitrogen). Samples were prepared by boiling for 5 minutes in NuPAGE[®] LDS sample loading buffer (4 ×). BenchMark[™] prestained protein ladder (Invitrogen) and biotinylated protein ladder (Cell Signaling Technology) were used for molecular weight comparison as appropriate.

Immunoblotting: Proteins were transferred from PAGE gels to Hybond[™]-ECL[™] nitrocellulose membranes (Amersham Biosciences) using a semi-dry electrophoretic transfer method. The membranes were blocked for 1 hour in BSA (5% in buffer C) before washing for 3 × 10 min in buffer C. The blots were probed using the appropriate antibody–HRP conjugate (anti-5 × His or NeutrAvidin[™]) at 1:5000 dilution in buffer C for 1 hour at room temperature. The membranes were then washed with buffer C for 3 × 5 min before detection using the enhanced chemiluminescence (ECL) kit according to the manufacturer's instructions (Amersham Biosciences).

Notes and references

EWT and RJL gratefully acknowledge BBSRC, MRC and Leverhulme Trust for financial support for this work, and EWT also acknowledges the award of a BBSRC David Phillips Research Fellowship. The authors are grateful to Prof. Deborah Smith (University of York), Dr. Tony Holder (NIMR) and Dr. Paul Bowyer (Stanford University, US) for their helpful discussions and suggestions.

^a Department of Chemistry, South Kensington Campus, Imperial College, London, SW7 2AZ, UK. Fax: +44 (0)20 75941139; Tel: +44 (0)20 75943752; E-mail: e.tate@imperial.ac.uk or

r.leatherbarrow@imperial.ac.uk

^b Chemical Biology Centre, South Kensington Campus, Imperial College, London, SW7 2AZ, UK.

† We have previously shown that a recombinant protein can be tagged *in vitro* using CaNMT. In this instance tagging of protein proceeds in a similar manner to tagging of the peptide substrates described above.¹³

‡ Note that in our previous work¹³ we deliberately depleted the lysate of this biotinylated protein prior to capture; this step may be omitted for convenience as in the present report.

1. H. Wang and X. Chen, *Front. Biosci.*, 2008, **13**, 1716-1732.

2. T. L. Foley and M. D. Burkart, *Curr. Opin. Chem. Biol.*, 2007, **11**, 12-19.

3. K. M. Marks and G. P. Nolan, *Nat. Methods*, 2006, **3**, 591-596.

4. J. E. Moses and A. D. Moorhouse, *Chem. Soc. Rev.*, 2007, **36**, 1249-1262.

5. N. J. Agard, J. M. Baskin, J. A. Prescher, A. Lo and C. R. Bertozzi, *ACS Chem. Biol.*, 2006, **1**, 644-648.

6. J. A. Prescher, D. H. Dube and C. R. Bertozzi, *Nature*, 2004, **430**, 873-877.

7. J. Xie and P. G. Schultz, *Nat. Rev. Mol. Cell Biol.*, 2006, **7**, 775-782.

8. T. Hohsaka and M. Sisido, *Curr. Opin. Chem. Biol.*, 2002, **6**, 809-815.

9. M. W. Rose, J. Xu, T. A. Kale, G. O'Doherty, G. Barany and M. D. Distefano, *Biopolymers*, 2005, **80**, 164-171.

10. U. T. Nguyen, J. Cramer, J. Gomis, R. Reents, M. Gutierrez-Rodriguez, R. S. Goody, K. Alexandrov and H. Waldmann, *ChemBioChem*, 2007, **8**, 408-423.

11. S. A. Slavoff, I. Chen, Y. A. Choi and A. Y. Ting, *J. Am. Chem. Soc.*, 2008.

12. I. S. Carrico, B. L. Carlson and C. R. Bertozzi, *Nat. Chem. Biol.*, 2007, **3**, 321-322.

13. W. P. Heal, S. R. Wickramasinghe, P. W. Bowyer, A. A. Holder, D. F. Smith, R. J. Leatherbarrow and E. W. Tate, *Chem. Commun.*, 2008, 480-482.

14. M. Kohn and R. Breinbauer, *Angew. Chem. Int. Ed. Engl.*, 2004, **43**, 3106-3116.

15. T. A. Farazi, G. Waksman and J. I. Gordon, *J. Biol. Chem.*, 2001, **276**, 39501-39504.

16. R. S. Gunaratne, M. Sajid, I. T. Ling, R. Tripathi, J. A. Pachebat and A. A. Holder, *Biochem. J.*, 2000, **348 Pt 2**, 459-463.

17. D. K. Giang and B. F. Cravatt, *J. Biol. Chem.*, 1998, **273**, 6595-6598.

18. R. C. Wiegand, C. Carr, J. C. Minnerly, A. M. Pauley, C. P. Carron, C. A. Langner, R. J. Duronio and J. I. Gordon, *J. Biol. Chem.*, 1992, **267**, 8591-8598.

19. D. A. Towler, S. P. Adams, S. R. Eubanks, D. S. Towery, E. Jackson-Machelski, L. Glaser and J. I. Gordon, *Proc. Natl. Acad. Sci. U. S. A.*, 1987, **84**, 2708-2712.

20. C. Panethymitaki, P. W. Bowyer, H. P. Price, R. J. Leatherbarrow, K. A. Brown and D. F. Smith, *Biochem. J.*, 2006, **396**, 277-285.

21. H. P. Price, M. R. Menon, C. Panethymitaki, D. Goulding, P. G. McKean and D. F. Smith, *J. Biol. Chem.*, 2003, **278**, 7206-7214.

22. R. J. Duronio, D. A. Rudnick, S. P. Adams, D. A. Towler and J. I. Gordon, *J. Biol. Chem.*, 1991, **266**, 10498-10504.

23. R. O. Heuckeroth, E. Jackson-Machelski, S. P. Adams, N. S. Kishore, M. Huhn, A. Katoh, T. Lu, G. W. Gokel and J. I. Gordon, *J. Lipid Res.*, 1990, **31**, 1121-1129.

24. B. Devadas, T. Lu, A. Katoh, N. S. Kishore, A. C. Wade, P. P. Mehta, D. A. Rudnick, M. L. Bryant, S. P. Adams, Q. Li and et al., *J. Biol. Chem.*, 1992, **267**, 7224-7239.

25. H. C. Hang, E. J. Geutjes, G. Grotenbreg, A. M. Pollington, M. J. Bijlmakers and H. L. Ploegh, *J. Am. Chem. Soc.*, 2007, **129**, 2744-2745.

26. D. D. Martin, G. L. Vilas, J. A. Prescher, G. Rajaiiah, J. R. Falck, C. R. Bertozzi and L. G. Berthiaume, *FASEB J.*, 2007.

27. P. W. Bowyer, R. S. Gunaratne, M. Grainger, C. Withers-Martinez, S. R. Wickramasinghe, E. W. Tate, R. J. Leatherbarrow, K. A. Brown, A. A. Holder and D. F. Smith, *Biochem. J.*, 2007, **408**, 173-180.

28. F. Eisenhaber, B. Eisenhaber, W. Kubina, S. Maurer-Stroh, G. Neuberger, G. Schneider and M. Wildpaner, *Nucleic Acids Res.*, 2003, **31**, 3631-3634.

29. S. Maurer-Stroh, B. Eisenhaber and F. Eisenhaber, *J. Mol. Biol.*, 2002, **317**, 523-540.

30. S. Maurer-Stroh, B. Eisenhaber and F. Eisenhaber, *J. Mol. Biol.*, 2002, **317**, 541-557.

31. W. H. Stafford, R. W. Stockley, S. B. Ludbrook and A. A. Holder, *Eur. J. Biochem.*, 1996, **242**, 104-113.

32. R. R. Rees-Channer, S. R. Martin, J. L. Green, P. W. Bowyer, M. Grainger, J. E. Molloy and A. A. Holder, *Mol. Biochem. Parasitol.*, 2006, **149**, 113-116.

33. E. W. Tate, P. W. Bowyer, K. A. Brown, D. F. Smith, A. A. Holder and R. J. Leatherbarrow, *Signal Transduction*, 2006, **6**, 160-163.

34. L. J. Knoll, D. R. Johnson, M. L. Bryant and J. I. Gordon, *Methods Enzymol.*, 1995, **250**, 405-435.

35. R. J. Duronio, E. Jackson-Machelski, R. O. Heuckeroth, P. O. Olins, C. S. Devine, W. Yonemoto, L. W. Slice, S. S. Taylor and J. I. Gordon, *Proc. Natl. Acad. Sci. U. S. A.*, 1990, **87**, 1506-1510.

36. J. H. Yoo, O. H. Cheng and G. E. Gerber, *Biochem. J.*, 2001, **360**, 699-706.

37. H. A. Van Valkenburgh and R. A. Kahn, *Methods Enzymol.*, 2002, **344**, 186-193.

38. P. A. Smith, B. C. Tripp, E. A. DiBlasio-Smith, Z. Lu, E. R. LaVallie and J. M. McCoy, *Nucleic Acids Res.*, 1998, **26**, 1414-1420.

39. A. E. Speers and B. F. Cravatt, *Chem. Biol.*, 2004, **11**, 535-546.

40. T. Sato, S. Fujita, M. C. Z. Kasuya, K. Hatanaka and T. Yamagata, *Chem. Lett.*, 2004, **33**, 580-581.