Supplementary Material (ESI) for Chemical Communications

Chemical Communications

Site-specific N-terminal labelling of proteins *in vitro* and *in vivo* using *N*-myristoyl transferase and bioorthogonal ligation chemistry

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ELECTRONIC SUPPLEMENTARY INFORMATION (4 Pages)

General Experimental: 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 a Bruker AM-400 spectrometer. 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 Unipoint™ 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 SecurityGuard™ cartridge system, C18, 4 × 3.0 mm (Phenomenex).

***ω*-Azido undecanoic acid (3):**[1] 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 $MgSO_4$. 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, $(CH_2)_6$), 1.55–1.72 (4H, m, $C\underline{H}_2CH_2CO_2H$ and $C\underline{H}_2CH_2N_3$), 2.36 (2H, t, J = 7.5 Hz, $C\underline{H}_2CO_2H$), 3.27 (2H, t, J = 7.0 Hz, CH_2N_3); 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 ($[(M - H]^-$).

***ω*-Azido undecanoic acid CoA thioester (4):**[1] To a stirring solution of *ω*-azido undecanoic acid (15 mg, 64 mmol) in dry THF (1.0 mL) under nitrogen was added a solution of CDI (12 mg, 77 mmol) 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 mmol) in aqueous $NaHCO_3$ (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 NH_4OAc pH 5.2 (0–5 min 0% MeCN, 5–10 min up to 50% MeCN, 10–15 min 50% MeCN). Detection was at 223 nm. The product was isolated as a white amorphous solid (11.4 mg, 17% yield). δ_H/ppm (400 MHz, D_2O) 0.70 (3H, s, CH_3), 0.84 (3H, s, CH_3), 1.08–1.29 (12H, m), 1.41–1.54 (4H, m), 2.34 (2H, bt, J = 6.0 Hz), 2.50 (2H, t, J = 7.2 Hz), 2.90 (2H, t, J = 5.9 Hz), 3.19 (2H, t, J = 6.9), 3.21–3.54 (5H, m), 3.77 (1H, bs), 3.95 (1H, bs), 4.16 (2H, bs), 4.51 (2H, bs), 4.61–4.82 (6H, m), 6.11 (1H, bd, J = 4.2 Hz), 8.31 (1H, s, Ar-H), 8.57 (1H, s, Ar-H); *m*/*z* (MALDI-TOF –), 1040 ($[(M + (NH_4^+)_3) - H]^-$).

Peptide Synthesis (H_2N-GLYVSRLFNRLFQKK-OH, 5):[2] *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(^{t}Bu), Tyr(^{t}Bu) 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/^{t}Bu 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),

[1] For previous syntheses, see B. Devadas *et al.*, *J. Biol. Chem.*, 1992, **267**, 7224-39.
[2] For a previous synthesis see Ref. 11a, main text.

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% H_2O 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 NH_4OAc 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 was obtained by lyophilisation as a white amorphous solid. *m*/*z* (MALDI-TOF +), 1870 ($[M+H]^+$).

Methyl 2-(diphenylphosphino)-4-(15-oxo-19-(2-oxohexahydro-1*H*-thieno[3,4-d]imidazol-4-yl)-4,7,10-trioxa-14-azanonadecylcarbamoyl)benzoate (Staudinger-Bertozzi Capture Reagent, 6): Biotin-PEG NovaTag™ resin (0.42 mmol g^{-1} loading, 286 mg, 0.12 mmol,) was deprotected (Fmoc) by treatment with 20% v/v piperidine in DMF before addition of 3-(diphenylphosphino)-4-(methoxycarbonyl)benzoic acid[3] (175 mg, 0.48 mmol), HATU (182 mg, 0.48 mmol) and DIPEA (2.0 mL) in DMF (2.0 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 H_2O (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 (47.6 mg, 50% yield). δ_H/ppm (400 MHz, CDCl3) 1.43 (2H, q, *J* = 7.6 Hz, biotin CH_2), 1.60–1.87 (8H, m, biotin CH_2 × 2 and $NHCH_2C\underline{H}_2CH_2O$ × 2), 2.19 (2H, dt, *J* = 1.6, 7.1 Hz, biotin $NHC(O)C\underline{H}_2$), 2.74 (1H, d, *J* = 12.9 Hz, biotin ½ × SCH_2), 2.91 (1H, dd, *J* = 4.9, 12.9, biotin ½ × SCH_2), 3.15 (1H, dt, *J* = 4.7, 7.4 Hz, $C\underline{H}_2NHC(O)CH_2$), 3.33 (2H, app. q, *J* = 5.9 Hz, biotin SCH), 3.41–3.66 (14H, m, 7 × PEG CH_2), 3.75 (3H, s, OCH_3), 4.33 (1H, dd, *J* = 4.6, 7.5 Hz, biotin $SCHC\underline{H}$), 4.52 (1H, dd, J = 4.9, 7.7 Hz, biotin $SCH_2C\underline{H}$), 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, PPh_2), 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, PPh_2), 32.67 (1P, s, $P(O)Ph_2$), 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 ($C_{41}H_{54}N_4O_8SP$, $[M + H]^+$, requires 793.3400).

IMPORTANT NOTE: DMSO stocks of **6** 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 **6** used in capture experiments, once a DMSO stock had been made up it was thoroughly de-gassed with helium, aliqotted into 20 μL portions and stored at –20 °C under $N_{2(g)}$.

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).

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 H_2O) 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), azidomyristoyl–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

[3] Synthesised according to T. Sato *et al.*, *Chem. Lett.* 2004, **33**, 580–581.

resultant solution was analysed by HPLC over a gradient of MeCN (0.1% TFA) in H_2O (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.

Recombinant CaNMT and PfARF1: CaNMT was cloned into pET11c (ampicillin resistance), expressed in *E. coli* and purified as described.[4] PfARF1 wild-type and G2A mutant proteins were cloned into pET28a (kanamycin resistance), expressed in *E. coli* and purified as reported previously.[5]

***In vitro* Assay:** Stock solutions containing PfARF1 (wild type and mutant, 100 μM in buffer B), CaNMT (10 μM in 50 mM Tris.HCl pH 8.0, 200 mM NaCl, 20% glycerol), acid–CoA **2** or **4** (1 mM in 10 mM NaOAc, pH 5.0–EtOH, 1:1), capture reagent **6** (10 mM in DMSO) and DTT (100 μM in H_2O) were prepared. Transfer reactions were carried out as follows: To buffer B (170 μL) was added DTT (4.2 μL), CaNMT (30 μL), acid CoA (6 μL) and PfARF1 (30 μL), the reactions were mixed thoroughly and incubated at 37 °C for 18 h. After sampling for PAGE, capture reagent **6** was added (200 μM final), the reactions mixed well and incubated for 2 hours at 37 °C. Finally, after sampling for PAGE, the reaction mixtures were added to NeutrAvidin™ agarose beads (washed thoroughly with buffer C to remove NaN_3) (1 mL settled beads/2 mg protein) and the reactions agitated in a carousel at room temperature for 30 min. The mixtures were spun at 10,000 rpm for 2 minutes in a bench-top centrifuge and the supernatant was sampled for PAGE. Volumes containing 2 μg protein were taken at each stage for each loading onto a gel.

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:** An aliquot of each cell lysate (prepared as above) containing 120 μg protein was depleted of endogenous biotinylated proteins as follows: an aliquot was added to 180 μL of settled NeutrAvidin™ agarose beads (washed thoroughly with buffer D to remove NaN_3) and the mixture mixed thoroughly before being agitated in a carousel at room temperature for 30 minutes. After this time the beads were spun at 10,000 rpm for 2 minutes in a bench-top centrifuge and the supernatant removed. The beads were washed with a small volume of buffer D and the washings were combined (to no more than 100 μL final volume). The total protein concentration was determined (~1 μg μL^{-1}). To an aliquot of the mixture containing 100 μg protein was added 5 μL DTT stock solution (100 mM in H_2O) and 2 μL capture reagent stock solution and the volume adjusted to 100 μL with buffer D if required. The resultant mixture was mixed thoroughly and incubated at 37 °C for 2 hours. After sampling for PAGE, an aliquot of the reaction mixture containing 30 μg of protein was added to 45 μL of settled NeutrAvidin™ agarose beads (washed thoroughly with buffer D to remove NaN_3) and the reactions agitated in a carousel at room temperature for 30 minutes. The mixtures were spun at 10,000 rpm for 2 minutes in a bench-top centrifuge and the supernatant was sampled for PAGE. Volumes containing *ca.* 5 μg protein were taken at each stage for each loading onto a gel, with protein loading adjusted to compensate for different levels of expression resulting in PfARF1 bands of equal intensity between wild type and G2A mutant on an anti-His-HRP Western blot.

SDS PAGE: Proteins were separated by SDS PAGE using NuPAGE® 12 × Bis-Tris gels 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.

[4] See reference 7 in main text.
[5] See reference 11a in main text.

Immunoblotting: Proteins were transferred from PAGE gels to Hybond™-ECL™ nitrocellulose membranes (Amersham Biosciences) using a semi-dry electrophoretic transfer method.[6] 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).

[6] **Using Antibodies: A Laboratory Manual.** E. Harlow and D. Lane. 1998. Coldspring Harbor Laboratory Press, Cold Spring Harbor, New York. ISBN 0-87969-544-7