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Synthesis and evaluation of 2-ethynyl-adenosine-5′-triphosphate as a chemical reporter for protein AMPylation†

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Protein AMPylation is a posttranslational modification (PTM) defined as the transfer of an adenosine monophosphate (AMP) from adenosine triphosphate (ATP) to a hydroxyl side-chain of a protein substrate. One recently reported AMPylator enzyme, Vibrio outer protein S (VopS), plays a role in pathogenesis by AMPylation of Rho GTPases, which disrupts crucial signaling pathways, leading to eventual cell death. Given the resurgent interest in this modification, there is a critical need for chemical tools that better facilitate the study of AMPylation and the enzymes responsible for this modification. Herein we report the synthesis of 2-ethynyl-adenosine-5′-triphosphate (**2eATP**) and its utilization as a non-radioactive chemical reporter for protein AMPylation.

Introduction

Posttranslational modifications (PTMs) are an evolutionary solution to expand the relatively limited number of amino acids used to synthesize all proteins found in nature. These modifications, which transform functional groups on amino acid side chains, can alter the function of a protein altogether.[1] For example, lysine acetylation converts the side-chain amino group into an amide functionality thereby affecting hydrogen bonding and ion pairing potential, features that can be important for conformational changes and protein–protein interactions. Additionally, phosphorylation of hydroxyl side chains is known to have a significant impact on cell signaling pathways. Many PTMs are readily reversed by complementary enzymes and can therefore serve as cellular switches *in vivo*. For instance, the phosphoester bonds formed by kinases during phosphorylation can be cleaved by phosphatases to reveal the original hydroxyl side chains.

Another lesser studied PTM, which is characterized by the transfer of an AMP group from ATP to hydroxyl side-chains of substrate proteins, is known as AMPylation (Fig. 1A). Although first reported more than 40 years ago, this modification went largely unstudied until recently.[2] The resurgent interest in this PTM is due to reports of adenyltranserases that are injected into human cells by bacterial pathogens.[3,4] Further investigations revealed that these enzymes actively participate in

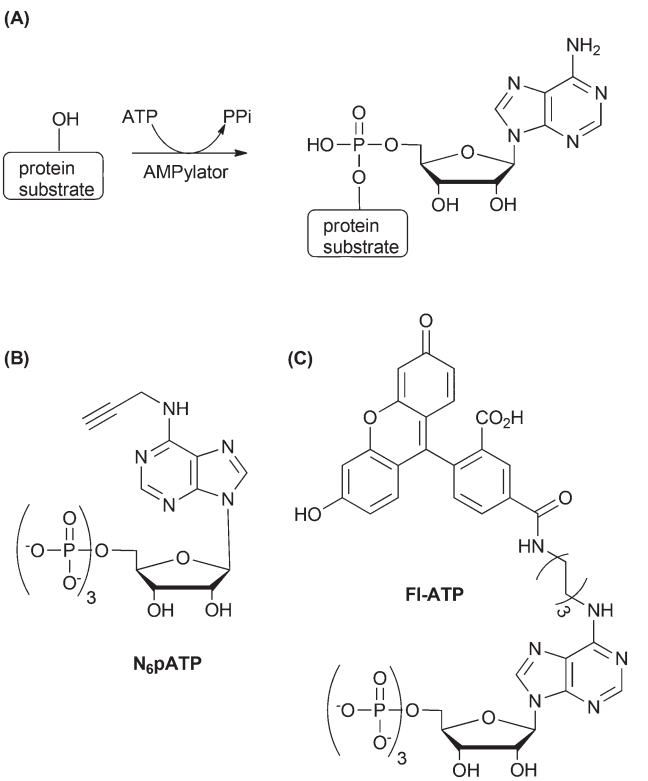

Fig. 1 Probing AMPylation. (A) AMPylator enzymes transfer AMP from ATP to hydroxyl side chains. (B) N^6-propargyl adenosine-5′-triphosphate (N^6pATP) and (C) N^6-(6-amino)hexyl-adenosine-5′-triphosphate-5-carboxyl-fluorescein (Fl-ATP) have been previously utilized as chemical reporters for protein AMPylation.

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pathogenesis.[5,6] Among the AMPylator enzymes reported to play roles in pathogenesis is Vibrio outer protein S (VopS) of *Vibrio parahaemolyticus*. Once released into host cells, this enzyme AMPylates a threonine residue in the Switch I region of Rho GTPases, such as Cdc42.[5] The addition of the AMP group causes disruptions in crucial interactions with downstream proteins, leading to cell rounding and eventual cell death. In addition to the discovery of AMPylator enzymes, a de-AMPylator enzyme has also recently been identified, suggesting that this PTM is dynamic and likely has significant *in vivo* roles.[7,8]

Given the emerging interest in understanding the roles of AMPylation, there is a need for tools that facilitate the study of this PTM. Radioisotopically labeled ATP can be used to visualize AMPylated protein, however this methodology requires specialize training and equipment. Furthermore, isotopic labeling does not aid in the selective isolation of modified substrates, thus identification still required robust proteomic analysis.[3,5,9,10] Such analysis of complex mixtures can be quite laborious and difficult, especially when the relative abundance of the analyte protein is low. For these reasons, the development of non-radioactive chemical tools that enable the study of AMPylation is quite advantageous.

In 2010, Hang and co-workers synthesized and utilized an ATP-alkyne analog as a clickable chemical reporter for protein AMPylation (Fig. 1B).[11] Like ATP, this analog is enzymatically transferred to substrate proteins, and the alkyne moiety enables protein visualization through Cu(I)-catalyzed azide–alkyne cycloaddition (CuAAC) reactions with azide-conjugated reporter tags. In 2012, Thompson and co-workers reported the use of a fluorescein-ATP conjugate (Fl-ATP) as a novel chemical tool to identify AMPylated substrates (Fig. 1C).[12] Substrate specificity studies detailed in the same report also suggest that, in addition to modifications at position 6, modifications at position 2 of the adenine ring may also be tolerated by the enzyme. Given the interest in studying this enzyme family, we set out to synthesize an ATP analog, substituted with an alkyne moiety at position 2, and evaluate its utility as a clickable chemical probe to study protein AMPylation (Fig. 2). We hypothesized that the structural differences (*i.e.*, substitution at position 2 rather than position 6) will yield an alternative tool that may be effective to study not only known AMPylator enzymes, but also as-of-yet unknown AMPylators with different tolerances for structural modifications to the ATP substrate. Herein we report the synthesis and *in vitro* evaluation of 2-ethynyl-adenosine-5′-triphosphate (**2eATP**) as a chemical reporter for protein AMPylation.

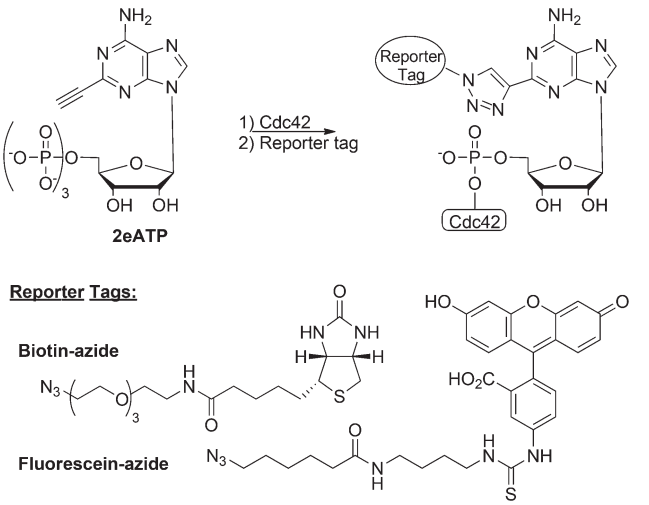


Fig. 2 Detection of modified protein substrates. Once **2eATP** is transferred to the substrate protein, detection of the modified protein can be facilitated by the addition of reporter tags *via* CuAAC reactions.

Results and discussion

Design of chemical reporter

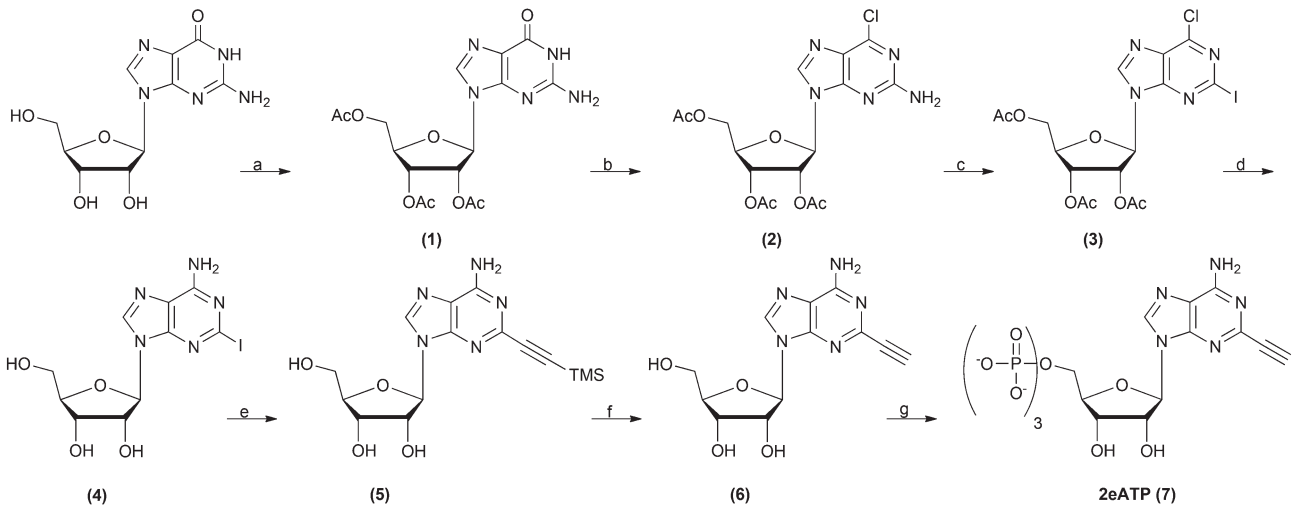
Traditional methods, such as radioisotopic labeling and mass spectrometry analysis of complex mixtures have certain limitations that can be overcome by fluorescein or biotin labeling of modified substrates. In addition to mitigating the need for radioactive reagents, use of these reporter tags can also enable isolation methods such as immuno-precipitation. An investigation into the substrate specificity of VopS indicated that modifications to the adenine ring at positions 2 and 6 would be tolerated by the enzyme.[12] Since the probes utilized previously were modified at position 6, we chose to explore the alternative position by installing an ethynyl group at position 2. Like the previously reported probe, the presence of the alkyne will enable the addition of azide-conjugated reporter tags *via* CuAAC reactions (Fig. 2). The addition of the alkyne to this position also confers alternative steric constraints, and therefore may prove useful in the study of subsequently discovered AMPylators.

Synthesis of 2-ethynyl-adenosine-5′-triphosphate (2eATP)

The synthesis of 2-ethynyl-adenosine-5′-triphosphate (**2eATP**) began from guanosine and proceeded in 7 total steps (Scheme 1). The three hydroxyl groups were first acetylated, followed by conversion to chloro-purine **2**. A Sandmeyer reaction was employed to replace the exocyclic amino group with an iodo group. Treatment of **3** with ethanolic ammonia yielded 2-iodoadensine *via* substitution of the chloride and concurrent removal of the three acetyl protecting groups. A Sonogashira reaction between **4** and TMS-protected acetylene, followed by deprotection of the silane, yielded 2-ethynyl-adenosine (**6**). Treatment of **6** with phosphoryl chloride, followed by bis(tri-*n*-butylaminium)pyrophosphate installed the triphosphate to yield **2eATP** (**7**) as the final product.

Fluorescent labeling of Cdc42

A major impetus for developing chemical probes for protein AMPylation is facile visualization of modified protein substrates. Although radioisotopically labeled ATP can be used, those experiments require radioactive reagents and equipment. The use of **2eATP** can mitigate these requirements when



Scheme 1 Synthesis of 2-ethynyl-ATP. Reagents and conditions: (a) Ac_2O, Et_3N, DMAP, DMF, rt, 1 h; (b) $POCl_3$, dimethylaniline, Et_4NCl, ACN, reflux, 10 min; (c) CH_2I_2, I_2, CuI, isopentyl nitrite, THF, reflux, 45 min; (d) NH_3, EtOH, 0 °C → rt, 48 h; (e) $PdCl_2(PPh_3)_2$, Et_3N, CuI, TMS-acetylene, ACN, rt, 20 min; (f) NH_3, MeOH, 0 °C → rt, 2.25 h; (g) $POCl_3$, bis(tri-n-butyl)ammonium pyrophosphate, $(MeO)_3PO$, 0 °C → rt.

used with a fluorescent reporter such as a fluorescein–azide. To test **2eATP**, both as a substrate and a fluorescent reporter for protein AMPylation, a known AMPylator enzyme (VopS) and decreasing amount of its cognate protein substrate (Cdc42Q61L) were incubated with **2eATP** at 30 °C for 1 h. Subsequent to the transfer reaction, the mixture was treated with fluorescein–azide under CuAAC conditions. The reaction mixtures were separated by SDS-PAGE and visualized fluorescently using a Typhoon 9410 imaging system. The results of this experiment show that **2eATP** is effectively transferred to Cdc42Q61L, as evidenced by the fluorescent bands; the limit of detection is approximately 5 pmol of modified protein (Fig. 3A). In addition to evaluating the transfer of **2eATP** to purified protein substrate, the selectivity of this transfer was also explored by conducting an analogous experiment in the presence of cell lysates from *E. coli* (Fig. 3B). Although there is a slight decrease in the sensitivity for detection, which is likely due to probe degradation by other ATP degrading enzymes, the transfer of **2eATP** to Cdc42Q61L is remarkably selective. This latter finding is consistent with the fact that VopS shows very little promiscuous activity. Note that at higher concentrations a second lower band, which is likely a proteolyzed fragment, becomes visible.

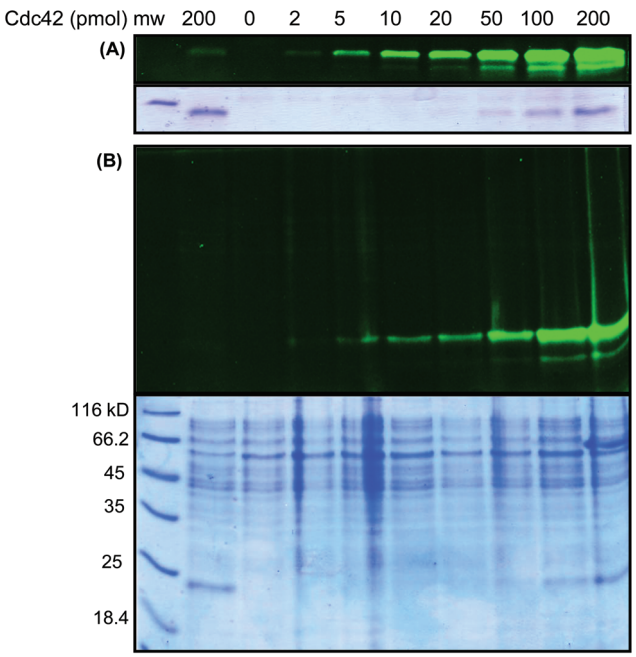


Fig. 3 Limit of detection and chemical selectivity. (A) Decreasing amounts of Cdc42 were treated with **2eATP** in the presence of VopS followed by a CuAAC reaction with fluorescein–azide; fluorescence (top), coomassie (bottom). (B) Selectivity of labeling: decreasing amounts of Cdc42 were added to *E. coli* cell lysates; fluorescence (top), coomassie. (bottom).

Biotin labeling of Cdc42Q61L

The use of click chemistry has found great utility for development of tools to study proteins. The ethynyl moiety of **2eATP** can facilitate not only the detection of modified protein by fluorescent imaging, but also through the addition of a biotin reporter tag. To demonstrate the ability to detect protein AMPylation using a biotin–azide conjugate, experiments analogous to those conducted with fluorescein–azide were undertaken. Decreasing amounts of Cdc42Q61L were incubated with VopS and **2eATP** at 30 °C for 1 h. Subsequent to the transfer reaction, biotin–azide was added *via* a CuAAC reaction, and the samples were separated by SDS-PAGE. Western blot analysis of these samples enabled visualization of the labeled protein to amounts as low as 10 pmol (Fig. 4A). In order to demonstrate the selectivity of biotin labeling, an analogous experiment was conducted in the presence of cell lysate from

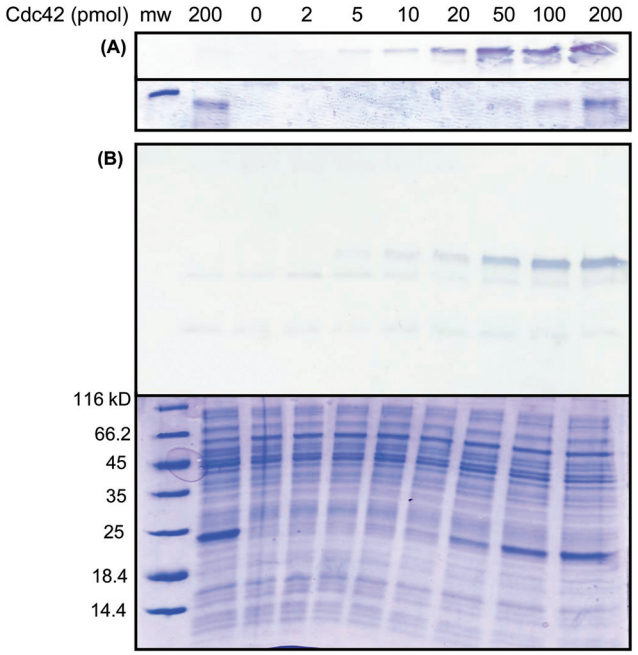


Fig. 4 Limit of detection and chemical selectivity. (A) Decreasing amounts of Cdc42 were treated with **2eATP** in the presence of VopS followed by a CuAAC reaction with biotin–azide; western blot (top), coomassie (bottom). (B) Selectivity of labeling: decreasing amounts of Cdc42 were added to *E. coli* cell lysates; western blot (top), coomassie. (bottom).

E. coli. Fig. 4B shows that a similar detection limit was achieved and that labeling was remarkably selective.

Conclusions

In conclusion, we have successfully synthesized **2eATP** and demonstrated that this ATP analog is effectively transferred by VopS to the cognate protein substrate, Cdc42Q61L. The alkyne moiety enables the post-transfer addition of reporter tags to the modified protein. These reporter tags, fluorescein–azide and biotin–azide, enable the visual detection of modified protein to quantities as low as 5 pmol. Given the growing interests in this PTM and the limited information available about its *in vivo* roles, **2eATP** should serve as a valuable tool to further explore the roles of protein AMPylation.

Experimental

All chemicals and solvents were of reagent grade and were purified and dried by standard methods prior to use. Bis(tri-*n*-butylammonium) pyrophosphate was prepared as previously described.[13] Biotin–azide was purchased from Sigma-Aldrich and fluorescein–azide was synthesize from 1,4-diaminobutane and FITC (isomer I) in a manor analogous to the previous report.[14] PGEX-GST-TEV-VopSΔ30 and pRoEX-HTa Cdc42Q61L were both expressed and purified as previously described.[12] Streptavidin-HRP antibody (cat# EMOR-03L) was purchased from VWR. ^{1}H, ^{13}C, and ^{31}P NMR spectra were collected on a Varian 500 MHz spectrometer and chemical shifts are reported (in ppm) relative to solvent peaks. Thin layer chromatography was conducted on pre-coated silica gel plates and column chromatography separations were conducted on silica gel (230–450 mesh). RP-HPLC separations were conducted on a Varian Prostar system with a microsorb column (21 × 250 mm).

2′,3′,5′-Triacetylguanosine (1)

To a mixture of guanosine (5 g, 17.7 mmol) and 4-diaminomethylpyridine (0.16 g, 1.3 mmol) in acetonitrile were added triethylamine (9.7 mL, 69.6 mmol) and acetic anhydride (6 mL, 63.5 mmol). After stirring for 45 min, methanol (3 mL, 74 mmol) was added and the mixture was stirred for an additional 10 min at rt. The reaction mixture was filtered, and the filtrate was concentrated under reduced pressure. Isopropyl alcohol (200 mL) was added to the resulting residue, and the mixture was refluxed for 10 min. After cooling to rt, the product was collected by filtration as a white powder (5.3 g, 12.9 mmol, 73%). ^{1}H NMR (500 MHz, 10% DMSO-d_6 in $CDCl_3$): δ 7.55 (s, 1H), 5.82 (d, J = 5 Hz, 1H), 5.77 (t, J = 5.5 Hz, 1H), 5.75 (br s, 2H), 5.58 (t, J = 5 Hz, 1H), 4.32 (dd, J = 11.5, 3.5 Hz, 1H), 4.26 (m, 1H), 4.22 (dd, J = 11.5, 5 Hz, 1H), 2.00 (s, 3H), 1.98 (s, 3H), 1.97 (s, 3H); ^{13}C NMR (125 MHz, 10% DMSO-d_6 in $CDCl_3$): δ 170.44, 169.48, 169.23, 157.59, 153.44, 150.65, 135.68, 118.00, 86.06, 79.51, 72.58, 70.31, 62.89, 20.57, 20.38, 20.27; HRMS ($C_{16}H_{20}N_5O_8^+$): calculated 410.1312, observed 410.1323.

2-Amino-6-chloro-9-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)purine (2)

Tetraethylammonium chloride (4.3 g, 26.2 mmol), *N*,*N*-dimethylaniline (1.66 mL, 13.1 mmol) and freshly distilled phosphoryl chloride (7.35 mL, 76.6 mmol) were added to 2′,3′,5′-triacetylguanosine (**1**) (5.3 g, 13 mmol) in dry acetonitrile (27 mL). After heating the mixture under reflux for 10 min, and the volatiles were immediately removed by vacuum distillation. The remaining residue was dissolved in chloroform (55 mL) and treated with cold water (36 mL) for 15 min at 0 °C for 15 min. The layers were separated, and the aqueous layer was further extracted with chloroform (3 × 20 mL). The organics were combined, washed with cold water (6 × 20 mL), 5% sodium bicarbonate (6 × 40 mL), and dried over sodium sulfate. The solution was filtered and added to isopropanol (27 mL) and the resulting mixture was evaporated under reduced pressure to a volume of ~18 mL. The resulting solution was stored at 4 °C overnight to crystalize. The product was isolated by filtration as a slightly yellow crystal (3.4 g, 7.9 mmol, 61%). ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.37 (s, 1H), 7.08 (br, 2H), 6.11 (d, J = 6 Hz, 1H), 5.88 (t, J = 6 Hz, 1H), 5.54 (dd, J = 4.5, 6 Hz, 1H), 4.40 (dd, J = 3.5, 11 Hz, 1H), 4.38 (m, 1H), 2.12 (s, 3H), 2.04 (s, 3H), 2.03 (s, 3H); ^{13}C NMR (125 MHz, DMSO-d_6): δ 170.14, 169.48, 169.33, 159.93, 153.71, 149.93, 141.30, 123.50, 84.87, 79.72, 71.91, 70.27, 62.98, 20.55, 20.42, 20.23; HRMS ($C_{16}H_{19}ClN_5O_7^+$): calculated 428.0973, observed 428.0981.

6-Chloro-2-iodo-9-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)-9*H*-purine (3)

Iodine (1.93 g, 7.6 mmol), diiodomethane (6.1 mL, 76 mmol), copper iodide (1.61 g, 8.46 mmol), and isopentyl nitrite (3.0 mL, 22.5 mmol) were added to 2-amino-6-chloro-9-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)purine (**2**) (3.25 g, 7.6 mmol) in dry THF (35 mL). The mixture was stirred under reflux for 45 min, cooled to rt, filtered and concentrated. The product (3.1 g, 5.7 mmol, 75%) was isolated by chromatography on silica (99 : 1 DCM/methanol). ^{1}H NMR (500 MHz, 10% DMSO-d_6 in $CDCl_3$): δ 8.82 (s, 1H), 6.30 (d, J = 4.5 Hz, 1H), 5.88 (t, J = 5.5 Hz, 1H), 5.63 (t, J = 5.5 Hz, 1H), 4.41 (m, 2H), 4.28 (dd, J = 5.5, 11.5 Hz, 1H), 2.11 (s, 3H), 2.06 (s, 3H), 2.01 (s, 3H); ^{13}C NMR (125 MHz, DMSO-d_6): δ 170.43, 169.75, 169.65, 152.36, 149.35, 146.29, 131.93, 118.57, 86.69, 80.05, 72.77, 70.00, 62.80, 20.83, 20.60, 20.49; HRMS ($C_{16}H_{17}ClIN_4O_7^+$): calculated 538.9831, observed 538.9841.

2-Iodoadenosine (4)

6-Chloro-2-iodo-9-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)-9*H*-purine (**3**) (0.77 g, 1.42 mmol) was added to dry ethanol (80 mL) and cooled to 0 °C. The reaction flask was sealed with a rubber septum and ammonia was bubbled through the solution until the starting material completely dissolved (~3 h). The reaction was allowed to warm to rt and stirred for an additional 48 h. Reaction progress was monitored by TLC (9 : 1 DCM/methanol). The solution was purged with air to remove excess ammonia and the solvent was removed under reduced pressure. Water (5 mL) was added to the residue, and the mixture was freeze dried. Water (5 mL) was added to the dried product and the product was collect by centrifugation as a white powder (0.46 g, 1.17 mmol, 83%). ^{1}H NMR (500 MHz. DMSO-d_6): δ 8.30 (s, 1H), 7.75 (br s, 2H), 5.80 (d, J = 6. Hz, 1H), 5.48 (br s, 1H), 5.23 (br s, 1H), 5.06 (br s, 1H), 4.52 (t, J = 5.5 Hz, 1H), 4.12 (t, J = 4 Hz, 1H), 3.94 (q, J = 4 Hz, 1H), 3.65 (dd, J = 4.5, 12.5 Hz, 1H), 3.54 (dd, J = 4, 12 Hz, 1H); ^{13}C NMR (125 MHz, DMSO-d_6): δ 155.98, 149.80, 139.49, 120.94, 119.06, 87.18, 85.85, 73.60, 70.56, 61.47; HRMS ($C_{10}H_{13}IN_5O_4^+$): calculated 394.0012, observed 394.0017.

2-[2-(Trimethylsilyl)ethynyl]-adenosine (5)

2-Iodoadenosine (**4**) (0.1 g, 0.25 mmol), copper iodide (3.8 mg, 0.02 mmol), $PdCl_2(PPh_3)_2$ (8.4 mg, 0.012 mmol) were added to triethylamine (2 mL) and acetonitrile (2 mL). The reaction mixture was stirred in the dark until all starting material was consumed (~20 min). (TLC 15% EtOH in chloroform). The volatiles were removed under reduced pressure and the product was purified on silica (eluting with chloroform, chloroform/ether, ether, 5% MeOH in ether, 10% MeOH in ether) and isolated as an off-white solid (77 mg, 0.21 mmol, 85%). ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.41 (s, 1H), 5.83 (d, J = 7 Hz, 1H), 4.43 (m, 1H), 4.15 (m, 1H), 3.92 (m, 1H), 3.65 (m, 1H), 3.57 (m, 1H), 0.21 (s, 9H); ^{13}C NMR (125 MHz, 10% DMSO-d_6 in $CDCl_3$): δ 155.36, 148.50, 145.06, 141.17, 119.73, 102.59, 91.22, 89.98, 87.01, 73.93, 71.41, 62.41, −0.62; HRMS ($C_{15}H_{22}N_5O_4Si^+$): calculated 364.1441, observed 364.1445.

2-Ethynyladenosine (6)

2-[2-(Trimethylsilyl)ethynyl]-adenosine (**5**) (71 mg, 0.2 mmol) was dissolved in MeOH (5 mL) and cooled to 0 °C. Ammonia was bubbled through the solution for 45 min. The reaction was allowed to warm to rt and stir for an additional 1.5 h. The solution was purged with air to remove excess ammonia and the sample was concentrated under reduced pressure to yield the title compound as a light brown solid (52 mg, 0.18 mmol, 90%). ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.42 (s, 1H), 7.51 (s, 2H), 5.85 (d, J = 6 Hz, 1H), 5.46 (br s, 1H), 5.18 (br s, 2H), 4.54 (t, J = 5.5 Hz, 1H), 4.42 (t, J = 4.5 Hz, 1H), 4.00 (s, 1H), 3.95 (m, 1H), 3.66 (dd, J = 4, 12 Hz, 1H), 3.54 (d, J = 12 Hz, 1H); ^{13}C NMR (125 MHz, 10% DMSO-d_6): δ 156.18, 149.48, 144.99, 141.23, 119.42, 88.03, 86.24, 83.42, 75.77, 74.05, 70.90, 61.89; HRMS ($C_{12}H_{14}N_5O_4^+$): calculated 292.1046, observed 292.1051.

2-Ethynyl-adenosine-5′-triphosphate (2eATP) (7)

2-Ethynyladenosine (**6**) (43.7 mg, 0.15 mmol) was dissolved in dry trimethylphosphate (0.5 mL) and the solution was stirred at 45 °C for 1.5 h. The solution was cooled to 0 °C and freshly distilled phosphoryl chloride (29 mg, 0.19 mmol) was added dropwise. Stirring was continued at 0 °C for 1 h, then a solution of bis(tri-*n*-butylammonium) pyrophosphate (1 M in DMF) (0.75 mL, 0.75 mmol) was added dropwise and stirring was continued at 0 °C. After 20 min, the reaction was allowed to warm to rt and stir for an additional 5 min. Triethylammonium bicarbonate buffer (1 M, pH 8.0, AcOH) (2 mL) was added and stirring was continued for 45 min at rt. The reaction mixture was diluted with water (20 mL) and freeze-dried. The residue was dissolved in water, and purified by RP-HPLC eluting with a gradient of 100 mM TEAA (pH 7) and acetonitrile (0–25%). ^{1}H NMR (500 MHz, D_2O): δ 8.49 (s, 1H), 5.95 (d, J = 6.5 Hz, 1H), 4.70 (m, 1H), 4.53 (m, 1H), 4.33 (m, 1H), 4.18 (m, 2H), 3.48 (s, 1H); ^{31}P NMR (202 MHz, D_2O): δ −10.88 (d, J = 19.8 Hz), −11.45 (d, J = 19.8 Hz), −23.30 (t, J = 19.8 Hz); HRMS ($C_{12}H_{15}N_5O_{13}P_3^-$): calculated 529.9879, observed 529.9914.

Limit of detection by fluorescence

Varying concentrations of Cdc42Q61L (0, 0.1, 0.25, 0.5, 1.0, 2.5, 5, and 10 μM) were added to assay buffer (20 mM HEPES pH 8.0, 100 mM NaCl, 1 mM DTT, and 5 mM $MgCl_2$), mixed with 10 μM **2eATP**, and 1 μM VopS to initiate the reaction. This reaction mixture was incubated at 30 °C for 1 h. The CuAAC reaction was initiated by adding 10 μM fluorescein–azide, 1 mM TCEP, 100 μM ligand (TBTA in 1 : 4 DMSO : butanol), and 2 mM $CuSO_4$ to the reaction mixture. The reactions were incubated in the dark for 1 h and quenched with SDS-loading dye. The mixtures were heated at 95 °C for 10 min and separated by SDS-PAGE. The gel fluorescence was measured using a Typhoon 9410 imaging system.

Limit of detection in a complex proteome by fluorescence

Purified VopS (1 μM) was incubated with *E. coli* lysate (25 μg total protein) in assay buffer (50 mM HEPES pH 8.0, 100 mM NaCl, 1 mM DTT, and 5 mM $MgCl_2$) along with 10 μM **2eATP** and varying concentrations of Cdc42Q61L (0, 0.1, 0.25, 0.5, 1, 2.5, 5, and 10 μM) for 1 h at 30 °C. The CuAAC reaction was initiated as described above. The samples were quenched with SDS-loading dye, heated at 95 °C, and separated by SDS-PAGE. Fluorescence was measured using a Typhoon 9410 imaging system.

Limit of detection by western blot

2eATP (10 μM) and VopS (1 μM) were added to assay buffer (20 mM HEPES pH 8.0, 100 mM NaCl, 1 mM DTT, and 5 mM $MgCl_2$) containing various concentrations of Cdc42Q61L (0, 0.1, 0.25, 0.5, 1.0, 2.5, 5, and 10 μM). The reaction was incubated at 30 °C for 1 h. Subsequently, the copper catalyzed click reaction was initiated by adding 10 μM biotin–azide, 1 mM TCEP, 100 μM ligand (TBTA in 1 : 4 DMSO : butanol), and 2 mM $CuSO_4$ to the reaction mixture. The reaction was incubated for 1 h before adding SDS-loading dye to quench the reaction. The mixtures were heated at 95 °C for 10 min and separated by SDS-PAGE in preparation for the western blot. The samples were transferred from the SDS-PAGE to a nitrocellulose membrane in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3) at 80 V for 60 min. The membrane was washed with TBS and TBST before incubating in blocking buffer (5% BSA in TBS) overnight at 4 °C. The membrane was washed again with TBS and TBST before adding the Streptavidin-HRP antibody (1 : 7500) in blocking buffer for 1 h at rt. The HRP signal was detected by applying a 1-step Ultra TMB solution (Pierce #37574).

Limit of detection in a complex proteome by western blot

E. coli lysate (25 μg total protein), **2eATP** (10 μM) and purified VopS (1 μM) were incubated in assay buffer (50 mM HEPES pH 8.0, 100 mM NaCl, 1 mM DTT, and 5 mM $MgCl_2$) with varying concentrations of Cdc42Q61L (0, 0, 0.1, 0.25, 0.5, 1, 2.5, 5, and 10 μM) for 1 h at 30 °C. The copper catalyzed click reaction with biotin–azide was initiated by adding 10 μM biotin–azide, 1 mM TCEP, 100 μM ligand (TBTA in 1 : 4 DMSO : butanol), and 2 mM $CuSO_4$ to the reaction mixture. The samples were quenched with SDS-loading dye, heated at 95 °C, and separated by SDS-PAGE. The samples were transferred to a nitrocellulose membrane and imaged as described above.

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