Synthesis of 7-aminocoumarin via Buchwald-Hartwig cross coupling for specific protein labeling in living cells

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Synthesis of 7-aminocoumarin via Buchwald-Hartwig cross coupling for specific protein labeling in living cells

Xin Jin, Chayasith Uttamapinant, and Alice Y. Ting

To enable minimally invasive studies of proteins in their native context, it is desirable to tag proteins with small, bright reporter groups. Recently, our lab described PRIME technology (for PRobe Incorporation Mediated by Enzymes) for such tagging[1-3]. An engineered variant of *Escherichia coli* lipoic acid ligase (LplA) is used to covalently attach a fluorescent substrate, such as 7-hydroxycoumarin, onto a 13-amino acid peptide recognition sequence (called LAP, for Ligase Acceptor Peptide) that is genetically fused to a protein of interest (POI) (Figure 1A). The targeting specificity is derived from the extremely high natural sequence specificity of LplA[4]. PRIME was used to label and visualize various LAP-tagged cytoskeletal and adhesion proteins in living mammalian cells.

One limitation of the 7-hydroxycoumarin probe used in our previous study is its pH-dependent fluorescence. The 7-OH substituent has a pKₐ of 7.5[5], and the fluorophore is only emissive in its anionic form. Proteins labeled by PRIME with 7-hydroxycoumarin (on the extracellular or luminal side) therefore cannot be visualized in acidic compartments of the cell such as the endosome (pH 5.5-6.5[6]), where >90% of 7-hydroxycoumarin is expected to be neutral and therefore non-fluorescent. This problem prevents the use of 7-hydroxycoumarin for imaging receptor internalization and recycling, for example.

A potential solution is to use 6,8-difluoro-7-hydroxycoumarin (Pacific Blue[5], Figure 1B), which has a reduced 7-OH pKₐ of 3.7. We found, however, that our engineered 7-hydroxycoumarin ligase, the W37V mutant of LplA, did not ligate an isosteric Pacific Blue substrate onto LAP efficiently[11]. It is likely that the ligase active site prefers neutral and hydrophobic substrates and therefore rejects Pacific Blue, which is predominantly anionic at physiological pH.

An alternative coumarin structure is 7-aminocoumarin, shown in Figure 1B. In contrast to 7-hydroxycoumarin and Pacific Blue, 7-aminocoumarin is expected to be both neutral and highly fluorescent at a wide range of pH values. We also predicted that it would be a substrate for W³⁷V LplA, since it is sterically similar to 7-hydroxycoumarin and is uncharged at physiological pH.

The synthesis of the 7-aminocoumarin substrate 6 required a novel route, however. Previous synthetic routes to 7-aminocoumarin derivatives have used either Pechmann[7] or Perkin[8] condensation. The Pechmann reaction condenses aminoresorcinol with β-ketoesters and unavoidably produces 4-alkyl substituted aminocoumarins. Based on our structure-activity studies, a substituent at the 4 position of coumarin is unlikely to be tolerated by LplA. The Perkin reaction condenses

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aminoresorcinaldehyde with malonic acid and requires N-alkylation to prevent spontaneous Schiff base formation. A resulting N-alkylated aminocoumarin would be considerably larger than 7-hydroxycoumarin and unlikely to be accepted by our coumarin ligase.

To access the simple, minimally bulky 7-aminocoumarin structure shown in Figure 1B, we devised a new synthetic route whose key feature is the palladium-catalyzed Buchwald-Hartwig cross coupling to convert the 7-OH group of 7-hydroxycoumarin into an unsubstituted primary aniline group. Our synthetic route (Scheme 1) begins with the 7-hydroxycoumarin substrate, which is protected as a methyl ester derivative. Triflic anhydride and pyridine were used to convert to 7-triflylcoumarin in 87% yield. The Buchwald-Hartwig cross coupling was then performed with benzophenone imine as a surrogate for ammonia. We used a catalytic combination of Pd(OAc)$_2$, BINAP, and Cs$_2$CO$_3$ previously designed to produce high coupling yields for electron-deficient aryl triflates and to reduce triflate hydrolysis. The benzophenone imine-coumarin adduct was obtained after gentle reflux with the catalyst system in THF in 70% yield. Benzophenone imine was then cleaved using acidic hydrolysis, which also hydrolyzed the methyl ester to give the final product, 7-aminocoumarin, in 76% yield. The overall yield for five synthetic steps was 42%.

We characterized the photophysical properties of 7-aminocoumarin and compared to the 7-hydroxycoumarin isostere. The excitation and emission maxima of 7-aminocoumarin are 380nm/444nm, similar to those of 7-hydroxycoumarin (386nm/448nm). The extinction coefficient of 7-aminocoumarin (18,400 M$^{-1}$cm$^{-1}$) is about half that of 7-hydroxycoumarin (36,700 M$^{-1}$cm$^{-1}$). As expected, 7-aminocoumarin fluorescence is fairly constant across the pH range 3-10, whereas 7-hydroxycoumarin fluorescence drops sharply at pH values < 6.5.

We next tested 7-aminocoumarin for ligation by LplA variants. Although W37V LplA is the best single mutant of LplA for 7-hydroxycoumarin ligation, we previously found that several other LplA single mutants also had coumarin ligation activity (W37I, G, A, S, and L). We therefore tested these LplA variants along with W37V LplA for 7-aminocoumarin ligation onto LAP. As with 7-hydroxycoumarin, W37V LplA was still the best among these for ligation of 7-aminocoumarin (data not shown). Figure 2C shows an HPLC analysis of this ligation reaction. The starred peak indicated in the HPLC trace was collected and analyzed by mass spectrometry to confirm its identity as the covalent adduct between 7-aminocoumarin and LAP (Figure S1). Negative controls with ATP omitted, or W37V LplA replaced by wild-type LplA, gave no ligation product.

We compared the kinetics of 7-aminocoumarin and 7-hydroxycoumarin ligation by W37V LplA. With 500 μM of coumarin probe (likely saturating the ligase active site), 78% LAP was converted to product with 7-aminocoumarin, compared to 46% conversion with 7-hydroxycoumarin, after a 55-minute reaction. A 2-fold difference in reaction extent was also observed at lower coumarin concentration (100 μM) after 70 minutes. At the reaction pH of 7.4, ~50% of 7-hydroxycoumarin is expected to be in the anionic form, whereas 7-aminocoumarin is neutral. The improved kinetics with 7-aminocoumarin likely reflects preferential binding of W37V LplA to neutral substrates.
7-aminocoumarin 6 was then used for PRIME labeling in living mammalian cells. Neurexin-1β, a transmembrane neuronal synapse adhesion protein, was fused to LAP at its extracellular N-terminus, and labeled with 7-aminocoumarin and W37V LplA added to the growth medium. Figure 3A shows cell images after 20 minutes of 7-aminocoumarin labeling on Human Embryonic Kidney (HEK) cells expressing LAP-neurexin-1β and a transfection marker (histone 2B fused to yellow fluorescent protein, or H2B-YFP). A point mutation in the LAP sequence (Lys→Ala), or replacement of W37V LplA with wild-type LplA, eliminated 7-aminocoumarin labeling.

To test the ability of 7-aminocoumarin to visualize neurexin in acidic endosomes, we incubated 7-aminocoumarin-labeled cells at 37°C for 20 minutes, to allow endocytic internalization of surface pools of neurexin-1β. Figure 3B shows the appearance of internal 7-aminocoumarin puncta in cells after this 20-minute internalization period. In contrast, cells similarly labeled with 7-hydroxycoumarin and then incubated, did not show internal fluorescence, due to quenching of 7-hydroxycoumarin fluorescence in acidic compartments.

We also tested 7-aminocoumarin for intracellular protein labeling. To deliver the probe across the cell membrane, we derivatized the carboxylic acid of 7-aminocoumarin 6 as an acetoxymethyl (AM) ester (Figure 4A). Upon entering cells, the AM ester is cleaved by endogenous esterases, releasing the parent 7-aminocoumarin 6 probe. To perform intracellular protein labeling, HEK cells were transfected with expression plasmids for both the coumarin ligase, W37V LplA, and a LAP fusion protein. 7-aminocoumarin-AM was incubated with cells for 10 minutes, then media was replaced over 60 minutes to allow endogenous anion transporters to clear excess unconjugated probe from the cytosol. Figure 4B shows specific labeling in cells expressing LAP-tagged yellow fluorescent protein (LAP-YFP), but not in neighboring untransfected cells. An alanine mutation in LAP sequence abolished 7-aminocoumarin labeling. To illustrate generality, we also labeled LAP-YFP targeted to the nucleus (LAP-YFP-NLS) and LAP fused to cytoskeletal protein β-actin.

In summary, to extend PRIME technology to imaging of proteins in acidic organelles while accommodating the steric and electronic constraints of our engineered coumarin ligase, we have designed a new fluorescent ligase substrate. 7-aminocoumarin was synthesized by a novel route, using palladium-catalyzed Buchwald-Hartwig cross coupling to efficiently convert the 7-OH substituent into a 7-NH₂ substituent. We demonstrated that 7-aminocoumarin could be site-specifically targeted to LAP fusion proteins by the coumarin ligase, both on the cell surface and inside living mammalian cells. PRIME tagging with this new probe represents one step in our ongoing effort to generalize PRIME for labeling of any cellular protein with diverse fluorophore structures.

**Experimental section**

**Synthetic methods**

All experiments were conducted using oven-dried glassware under N₂ atmosphere and at ambient temperature (20-25°C) unless otherwise specified. All other chemicals were purchased from Alfa Aesar or Aldrich and used without further purification. ¹H-NMR, ¹³C-NMR and ¹⁹F-NMR spectra were recorded on a Varian Mercury spectrometer and referenced to the solvent. Chemical shifts are reported as δ values (ppm) referenced
to the solvent residual signals: CD$_3$OD, $\delta$-H 3.31 ppm, $\delta$-C 49.15 ppm; CD$_2$Cl$_2$, $\delta$-H 5.32 ppm, $\delta$-C 54.00 ppm; D$_2$O, $\delta$-H 4.80 ppm; CF$_3$COOH for $^{19}$F-NMR, $\delta$-F -78.50 ppm.

Data for $^1$H NMR are reported as follows: chemical shift ($\delta$ ppm), multiplicity (s = singlet, brs = broad singlet, d = doublet, t = triplet, q = quartet, m = multiplet), integration, coupling constant J (Hz). High-resolution mass spectra were obtained on a Bruker Daltonics APEXIV 4.7 Tesla Fourier transform mass spectrometer. Flash column chromatography was performed with 70-230 mesh silica gel.

Synthesis of 7-hydroxycoumarin 2
To a solution of 7-hydroxycoumarin-3-carboxylic acid succinimidyl ester 1 (50 mg, from AnaSpec) in anhydrous DMF (0.5 mL) was added 5-aminovaleric acid (55 mg) and anhydrous triethylamine (0.1 mL). The reaction proceeded for 4 hours at 25°C in the dark. The mixture was diluted with ethyl acetate (10 mL) and 1 M HCl (10 mL). Layers were separated, and the aqueous layer was extracted with ethyl acetate (15 mL x 3). The combined organic layer was washed by water and brine. The organic phase was dried over Na$_2$SO$_4$ and concentrated in vacuo. The residue was purified by preparatory thin-layer chromatography (silica gel, 90:5:5 EtOAc:MeOH:acetic acid) to give 2 as yellow solid (48 mg, 98%). High-resolution ESI-MS characterization gave 306.0983 observed; 306.0972 calculated for [M+H]$^+$.

$^1$H-NMR (400 MHz, CD$_3$OD, 25°C): 8.75 (s, 1H), 7.66 (d, 1H, J=8.7), 6.87 (dd, 1H, J=2.1, 8.6), 6.76 (d, 1H, J=1.9), 3.54 (m, 2H, CH$_2$), 2.31 (t, 2H, CH$_2$), 1.68 (m, 4H, CH$_2$).

$^{13}$C-NMR (500 MHz, CD$_3$OD, 25°C): δ 175.4, 165.3, 163.1, 157.9, 149.5, 132.5, 115.6, 114.1, 112.5, 103.1, 52.2, 40.7, 34.3, 29.7, 22.8.

Synthesis of 7-hydroxycoumarin methyl ester 3
To a solution of 2 (5 mg) in MeOH (1 mL) was added 1 M HCl solution in water (0.1 mL). The reaction proceeded for 24 hours at 25°C. Purification by flash column chromatography (silica gel, 20:80 hexanes:EtOAc) afforded 3 (5 mg, 93%) as a yellow solid. High-resolution ESI-MS characterization 320.1139 observed; 320.1129 calculated for [M+H]$^+$.

$^1$H-NMR (500 MHz, CD$_2$Cl$_2$, 25°C): 8.89 (s, 1H), 7.85 (d, 1H, J=8.7), 7.38 (d, 1H, J=2.1), 7.33 (dd, 1H, J=2.0, 8.7), 3.64 (s, 3H, CH$_3$), 3.45 (m, 2H, CH$_2$), 2.35 (t, 2H, CH$_2$), 1.68 (m, 4H, CH$_2$).

$^{13}$C-NMR (500 MHz, CD$_2$Cl$_2$, 25°C): δ 174.1, 161.1, 160.9, 155.3, 152.6, 147.25, 132.2, 119.2, 119.1, 117.9, 115.3, 110.7, 51.9, 39.9, 34.0, 29.4, 22.8.

Synthesis of 7-trifluoromethylsulfonylcoumarin methyl ester 4
To a solution of 3 (38 mg, 0.12 mmol) in anhydrous dichloromethane (5 mL) and anhydrous pyridine (0.1 mL) at 0°C was slowly added trifluoromethanesulfonic anhydride (30 μL, 0.18 mmol). The resulting mixture was stirred at room temperature for 2 h. The reaction was quenched with brine and diluted with ethyl acetate (10 mL). Layers were separated, and the aqueous layer was extracted with ethyl acetate (10 mL x 3). The combined organic phase was dried over Na$_2$SO$_4$ and concentrated in vacuo to afford 4 (39 mg, 87%) as brown solid. The product was used in the next reaction without further purification. ESI-MS characterization gave 452.0611 observed; 452.0621 calculated for [M+H]$^+$.

$^1$H-NMR (500 MHz, CD$_2$Cl$_2$, 25°C): 8.89 (s, 1H), 7.85 (d, 1H, J=8.7), 7.38 (d, 1H, J=2.1), 7.33 (dd, 1H, J=2.0, 8.7), 3.64 (s, 3H, CH$_3$), 3.45 (m, 2H, CH$_2$), 2.35 (t, 2H, CH$_2$), 1.68 (m, 4H, CH$_2$). $^{13}$C-NMR (500 MHz, CD$_2$Cl$_2$, 25°C): δ 174.1, 161.1, 160.9, 155.3, 152.6, 147.25, 132.2, 119.2, 119.1, 117.9, 115.3, 110.7, 51.9, 39.9, 34.0, 29.4, 22.8. $^{19}$F-NMR (300 MHz, CD$_2$Cl$_2$, 25°C): δ -72.98.
Synthesis of 7-diphenylmethyleneaminocoumarin methyl ester 5

An oven-dried flask was charged with (R)-(+-)BINAP (11 mg, 0.02 mmol), palladium(II) acetate (3 mg, 0.2 mmol), 4 (86 mg, 0.2 mmol) and cesium carbonate (164 mg, 0.5 mmol) and then purged with nitrogen. Benzophenone imine (46 mg, 0.025 mmol) and THF (5 mL) was added and the mixture was stirred at reflux under nitrogen for 4 hours. The mixture was cooled to room temperature, filtered, and concentrated. The yellow residue was purified by column chromatography (silica gel, 95:5 → 50:50 hexanes:EtOAc) to give 5 (53 mg, 70%) as a yellow solid. ESI-MS characterization gave 483.1932 observed; 483.1914 calculated for [M+H]+.

1H-NMR (500 MHz, CD3OD, 25°C): 8.75 (s, 1H), 7.73 (d, 1H, J=8.7), 7.2-7.7 (m, 10H) 6.86 (dd, 1H, J=1.9, 8.6), 6.79 (s, 1H), 3.60 (s, 3H, CH3), 3.42 (m, 2H, CH2), 2.37 (t, 2H, CH2), 1.66 (m, 4H, CH2).


Synthesis of 7-aminocoumarin 6

To a stirring solution of 5 (10 mg, 21 mmol) in 1:1 THF:water (10 mL) was added 1M HCl (0.5 mL). The reaction was stirred at 25°C for 48 hours, then concentrated in vacuo. The yellow residue was purified by column chromatography (silica gel, 94:5:1 EtOAc:MeOH:NH4OH) to afford 6 as a light yellow solid (5 mg, 76%). ESI-MS characterization gave 303.0973 observed; 303.0986 calculated for [M-H]-.

1H-NMR (500 MHz, D2O, 25°C): 8.30 (s, 1H), 7.36 (d, 1H, J=8.3), 6.66 (d, 1H, J=8.6), 6.40 (s, 1H), 3.36 (m, 2H, CH2), 2.29 (t, 2H, CH2), 1.66 (m, 4H, CH2).

13C-NMR (500 MHz, CD3OD, 25°C): δ 181.8, 164.6, 163.3, 158.4, 148.9, 132.2, 113.5, 109.7, 109.5, 98.4, 39.8, 38.1, 29.8, 24.5. λmax (ε) = 380 nm (18,400 M-1 cm-1) in pH 7 phosphate buffer.

Synthesis of 7-aminocoumarin-AM

To a stirring solution of 7-aminocoumarin 6 (3 mg, 9 μmol) in anhydrous acetonitrile (1 mL) was added silver(I) oxide (6 mg, 30 μmol) followed by acetoxyethyl bromide (1.5 μL, 15 μmol). The reaction was stirred at 25°C for 12 hours, then concentrated in vacuo. The yellow residue was purified by column chromatography (silica gel, 8:1 EtOAc:hexane) to afford 7-aminocoumarin-AM as a light yellow solid (3 mg, 81% yield). ESI-MS characterization gave 377.1348 observed; 377.1343 calculated for [M+H]+.

1H-NMR (300 MHz, CDCl3, 25°C): 8.39 (s, 1H), 7.28 (d, 1H, J=8.7), 6.70 (dd, 1H, J=8.6, 2.4), 6.45 (d, 1H, J=2.4), 5.72 (s, 2H), 3.34 (m, 2H, CH2), 2.31 (t, 2H, CH2), 2.09 (s, 3H, CH3), 1.70 (m, 4H, CH2).

7-Aminocoumarin and 7-hydroxycoumarin pH profiles (Figure 2B)

Fluorescence emission was recorded for 150 μM solutions, using a TECAN Safire Microplate Reader and a plastic transparent-bottomed 384-well plate (Greiner). pH 3-6 buffers were prepared by mixing different ratios of 0.1M acetic acid and 0.1M sodium acetate-trihydrate solutions. pH 7-10 buffers were prepared by mixing different ratios of 0.1M Na2HPO4 and either 0.1M HCl (for pH 7-9 buffers) or 0.1M NaOH (for pH 10 buffer). Final pH adjustments in all buffer solutions were made by adding small amount of 1M HCl or 1M NaOH.
In vitro 7-aminocoumarin ligation reactions (Figures 2C and S2)

For Figure 2C, reactions were assembled as follows: 2 μM LplA enzyme, 150 μM LAP2 synthetic peptide (sequence: GFEIDKVWYDLDA[16]), 500 μM 7-aminocoumarin 6 probe, 5 mM ATP, and 5 mM Mg(OAc)₂ in 25 mM Na₂HPO₄ pH 7.2. The reaction mixture was incubated at 30 °C for 2 hours and quenched with EDTA (final concentration 100 mM). The mixture was analyzed on a Varian Prostar HPLC using a reverse-phase C18 Microsorb – MV 100 column (250 × 4.6 mm). Chromatograms were recorded at 210 nm. We used a 10-minute gradient of 30–60% acetonitrile in water with 0.1% trifluoroacetic acid under 1 mL/minute flow rate. LAP2 had a retention time of 7 minutes; after ligation to 7-aminocoumarin, the retention time increased to 9 minutes.

For Figure S2, 2 μM W37V LplA and 500 μM coumarin probe were used in one case. Aliquots from the reaction were collected and quenched with EDTA over 55 minutes. For the other case, 1 μM W37V LplA and 100 μM coumarin probe were used, and aliquots were collected and quenched over 70 minutes. After HPLC analysis, percent product conversions were calculated by dividing the product peak area by the sum of (product + starting material) peak areas.

Mass spectrometric analysis of peptides (Figure S1)

Starred peaks from Figure 2C were manually collected and injected into an Applied Biosystems 200 QTRAP mass spectrometer. The flow rate was 3 μL/minute and mass spectra were recorded under the positive-enhanced multi-charge mode.

Mammalian cell culture

Human Embryonic Kidney (HEK) cells were cultured in Dulbecco’s modified Eagle medium (DMEM; Cellgro) supplemented with 10% v/v fetal bovine serum (PAA Laboratories). For imaging, cells were plated as a monolayer on glass coverslips. Adherence of HEK cells was promoted by pre-coating the coverslip with 50 μg/mL fibronectin (Millipore). All cells were maintained at 37 °C under 5% CO₂.

PRIME cell surface labeling (Figure 3)

HEK cells were transfected at ~70% confluency with expression plasmids for LAP4.2[16]-neurexin-1β (400 ng for a 0.95 cm² dish) and H2B-YFP (100 ng) using Lipofectamine 2000 (Invitrogen). 18 hours after transfection, cells were treated with 10 μM W37V LplA enzyme, 200 μM coumarin probe, 1 mM ATP, and 5 mM Mg(OAc)₂ in cell growth media for 20 minutes at room temperature. After removal of excess labeling reagents by replacing media 2-3 times, cells were immediately imaged, or incubated at 37°C for 20 minutes to allow cell surface protein turnover.

PRIME intracellular labeling (Figure 4)

HEK or HeLa cells were transfected with expression plasmids for W37V LplA (20 ng) and LAP substrate (LAP2-YFP, LAP2-YFP-NLS, or LAP2-β-actin; 400 ng) using Lipofectamine 2000. 18 hours after transfection, cells were treated with 20 μM 7-aminocoumarin-AM in serum-free DMEM for 10 minutes at 37 °C. Excess coumarin probe was removed by washing cells with cell growth media 4 times, for 15 minutes each time. Cells were imaged live thereafter.
Fluorescence imaging

Cells were imaged in Dulbecco’s Phosphate Buffered Saline (DPBS) in confocal mode. We used a Zeiss Axiovert 200M inverted microscope with a 40x oil-immersion objective. The microscope was equipped with a Yokogawa spinning disk confocal head, a Quad-band notch dichroic mirror (405/488/568/647), and 405 (diode), 491 (DPSS), and 561 nm (DPSS) lasers (all 50 mW). 7-Aminocoumarin (405 laser excitation, 445/40 emission), YFP (491 laser excitation, 528/38 emission), and DIC images were collected using Slidebook software. Fluorescence images in each experiment were normalized to the same intensity ranges. Acquisition times ranged from 10–1000 milliseconds.

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References


**Legends for figures and schemes**

**Figure 1.** PRIME (PRobe Incorporation Mediated by Enzymes) for site-specific labeling of proteins of interest (POIs) with coumarin fluorophores. A) Labeling scheme. Coumarin ligase is the W37V mutant of *E. coli* lipoic acid ligase (LplA) [REF PNAS 2010]. LAP is a 13-amino acid recognition sequence for LplA [REF JACS 2009]. B) Coumarin substrates for coumarin ligase. 7-Hydroxycoumarin and Pacific Blue substrates have been previously described [REF]. 7-Aminocoumarin was synthesized and characterized in this work.

**Scheme 1.** Synthesis of the 7-aminocoumarin substrate for coumarin ligase.

**Figure 2.** *In vitro* characterization of 7-aminocoumarin ligation. A) Fluorescence excitation and emission spectra for 7-aminocoumarin 6. B) pH profile for 7-amino and 7-hydroxycoumarins. Fluorescence intensity ratio (I at λ<sub>max</sub> divided by maximal fluorescence intensity I<sub>max</sub>) is plotted against pH. Each measurement was performed in triplicate. Error bars, ± 1 s.d. C) HPLC traces showing 7-aminocoumarin 6 ligation onto LAP peptide, catalyzed by W<sup>37V</sup>LplA. The starred peaks were collected and analyzed by mass spectrometry (Figure S1). Negative controls (black traces) are shown with ATP omitted or wild-type LplA in place of W<sup>37V</sup>LplA.

**Figure 3.** 7-Aminocoumarin ligation to LAP-neurexin on the surface of living mammalian cells. A) HEK cells expressing LAP-neurexin-1β were labeled with 7-aminocoumarin and purified W<sup>37V</sup>LplA added to the culture media. Negative controls are shown with a Lys→Ala mutation in LAP (second row), and with W<sup>37V</sup>LplA replaced by wild-type LplA (third row). H2B-YFP is the YFP transfection marker. Scale bars, 20 μm. B) Visualization of internalized LAP-neurexin using 7-aminocoumarin. Top row, HEK cells expressing LAP-neurexin were labeled as in (A), then incubated at 37°C for 20 minutes prior to imaging. Bottom row shows the same experiment using 7-hydroxycoumarin instead of 7-aminocoumarin. Scale bars, 10 μm.

**Figure 4.** 7-Aminocoumarin ligation inside living mammalian cells. (A) Structure of membrane-permeant 7-aminocoumarin-acetoxymethyl (AM) ester, and the deprotection
reaction catalyzed by endogenous esterases. (B) Specific labeling of LAP in the cytosol, nucleus, and on a cytoskeletal protein (actin). Labeling was performed for 10 minutes with co-expressed W37V LplA. A negative control is shown with a Lys→Ala mutation in LAP (second column). NLS = nuclear localization sequence. Scale bars, 10 μm.

**Text suggestions for the table of contents**

We report a synthesis of a pH-insensitive blue fluorophore 7-aminocoumarin based on palladium-catalyzed Buchwald-Hartwig cross coupling. 7-aminocoumarin can be used to tag recombinant proteins on the cell surface and inside living cells via PRIME (PRobe Incorporation Mediated by Enzymes), and unlike 7-hydroxycoumarin, can be visualized in acidic organelles such as endosomes.
Figure 1

A)

13-amino acid LAP → coumarin probe → coumarin ligase, ATP

B)

7-aminocoumarin
7-hydroxycoumarin
Pacific Blue
Scheme 1

1. \( \text{Et}_2\text{N}, \text{DMF, 98\%} \)

2. \( \text{HCl, MeOH, 93\%} \)

3. \( \text{Tf}_2\text{O, pyridine, CH}_2\text{Cl}_2, 87\% \)

4. \( \text{Pd(OAc)}_2, \text{BINAP, Cs}_2\text{CO}_3, \text{THF, reflux, 70\%} \)

5. \( \text{cat. HCl in 1:1 THF:water, 76\%} \)

Overall yield 42\%
Figure 2

A) 

B) 

C)
Figure 3

A) 
- **Coumarin**
- **YFP marker + DIC**
- LAP(K→A)
- Wild-type LgI1A

B) 
- **Coumarin**
- **Coumarin**
- 7-NH₂
- 7-OH
- 37°C 20 min
Figure for table of contents

via Buchwald-Hartwig cross coupling

Peptide tag
Protein of interest

X = OH
X = NH$_2$

Coumarin imaging of proteins in acidic cellular compartments