

**A highly sensitive protocol for microscopy of alkyne lipids and
fluorescently tagged or immunostained proteins**

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Supplementary Information for synthesis of reagents

(AP6Btn, AP10Btn, APpic2Btn, and ApicSBDP)

**and troubleshooting guide for the labeling of alkyne lipids
with azide reporters for lipid imaging**

Synthesis of azide detection reagents

AP6Btn and AP10Btn

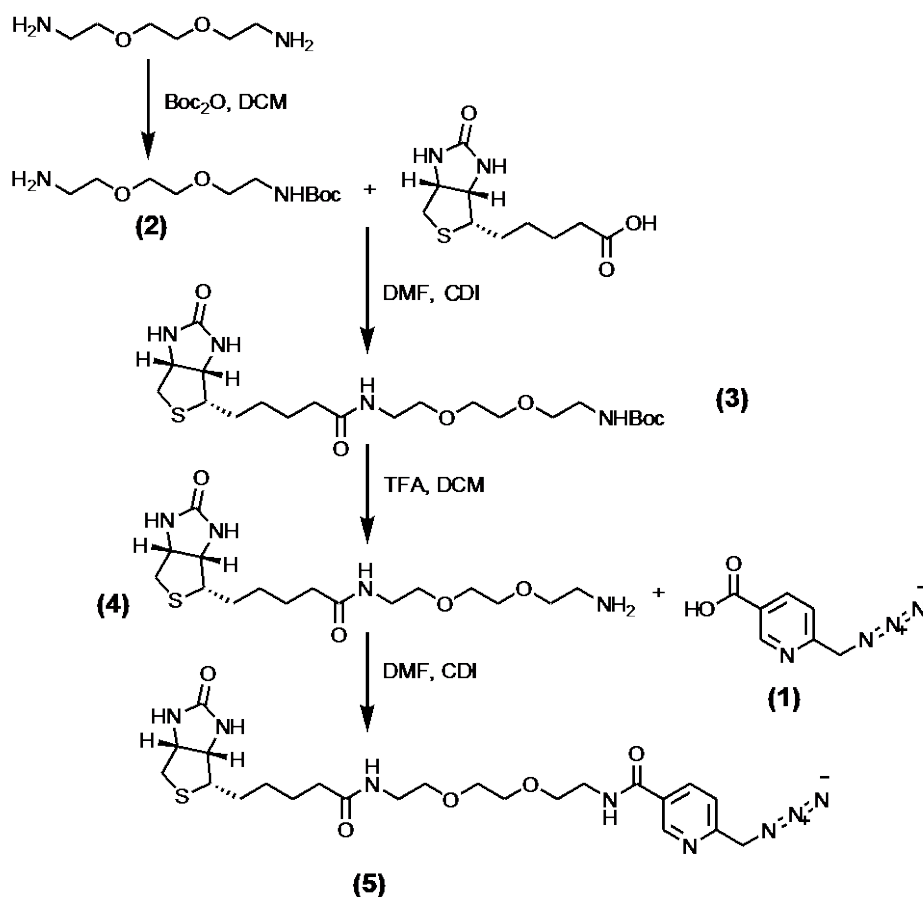
In a 50 ml roundbottom flask, biotin (122 mg, 0.5 mmol, 1 eq) was dissolved under Ar in 3 ml dry DMF at 80 °C. Then 1,1-carbonyldiimidazole (CDI) was added (89 mg, 0.55 mmol, 1 eq) and the mixture stirred at RT for 4 h. A solution of the azido-PEG-amine linker (0.55 mmol, *O*-(2-aminoethyl)-*O'*-(2-azidoethyl)pentaethylene glycol, Sigma Aldrich #76172, or *O*-(2-aminoethyl)-*O'*-(2-azidoethyl)nonaethylene glycol, Sigma Aldrich #77787, respectively) in dimethylformamide (DMF) was added and the mixture stirred at RT for 20 h. The solvents were evaporated, the reaction mixture was extracted with saturated aqueous sodium hydrogen carbonate and the product extracted with chloroform. The crude product was purified by column chromatography with CHCl₃/MeOH/H₂O 65/25/4. 238 mg waxy, nearly white substance (0.41 mmol, 82%) resulted for AP6Btn. AP10Btn was obtained as 309 mg (0.41 mmol, 82%) waxy, nearly white solid.

¹H-NMR AP6Btn (400 MHz, CDCl₃): 6.65 (t, 1H, amide-NH), 6.19 (s, 1H, NH), 5.26 (s, 1H, NH), 4.48 and 4.30 (2 x m, 2H), 3.7-3.3 (m, 28H), 3.13 (m, 1H), 2.87-2.91 (dd, 1H), 2.74-2.71 (d, 1H), 2.21 (m, 2H), 1.8-1.6 (m, 4H), 1.5-1.4 (m, 2H).

¹H-NMR AP10Btn (400 MHz, CDCl₃): 6.66 (t, 1H, amide-NH), 5.96 (s, 1H, NH), 5.10 (s, 1H, NH), 4.48 and 4.30 (2 x m, 2H), 3.7-3.3 (m, 44H), 3.14 (m, 1H), 2.86-2.94 (dd, 1H), 2.73-2.70 (d, 1H), 2.21 (m, 2H), 1.8-1.6 (m, 4H), 1.5-1.4 (m, 2H).

APpic2Btn

The picolyl-containing azide 6-azidomethylbenzoic acid (**1**) was synthesized according to a published procedure [1] and used in the synthesis of APpic2Btn.



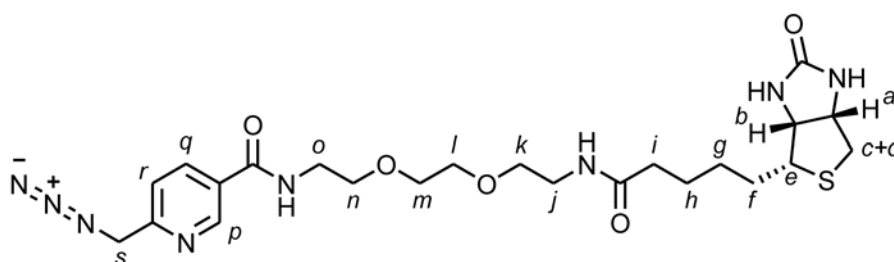
Synthesis of APpic2Btn (**5**).

Mono-protection of diamino-3,6-dioxaoctane [2]: Diamino-3,6-dioxaoctane (5.92 g, 5.92 ml, 40 mmol) was dissolved in dichloromethane (DCM, 400 ml), cooled to 0 °C and di-*tert*-butyl dicarbonate (Boc_2O , 1.31 g, 6 mmol, 0.15 eq) was added. The mixture was stirred for 5 h, warmed to RT and stirred for another 16 h. The organic phase was washed with water until complete removal of the diamine educt. After drying (MgSO_4) and concentration under vacuum, pure 1-Boc-amino-8-amino-3,6-dioxaoctane (**2**) (804 mg, 3.2 mmol, 54% regarding Boc_2O) was obtained.

$^1\text{H-NMR}$ (400 MHz, CDCl_3): 3.5 and 3.6 (m, 8H), 5.1 (br s, 1H), 3.30 (br, 2H), 2.85 (t, 2H), 1.42 (s, 9H).

Coupling of **(2)** to biotin (Btn-PEG2-NHBoc, **(3)**): Biotin (122 mg, 0.5 mmol) was dissolved under Ar in 3 ml DMF at 80 °C. Then CDI was added (214 mg, 1.32 mmol) and the mixture stirred at RT for 4 h. To this was added a solution of **(2)** (124 mg, 0.5 mmol) in 1 ml DMF and the mixture stirred at RT for 16 h. The solvent was removed *in vacuo* and the residue purified by silica gel chromatography (chloroform/methanol/water 65/25/4) to yield 174 mg **(3)** (0.38 mmol, 76%, $R_f = 0.58$).

¹H-NMR (400 MHz, CDCl₃, for atom labels see scheme below): 4.48 (m, 1H, H_b), 4.31 (m, 1H, H_a), 3.7-3.4 (m, 12H, H_j, H_k, H_l, H_m, H_n, H_o), 3.14 (m, 1H, H_e), 2.9 (m, 1H, H_c), 2.73 (m, 1H, H_d), 2.22 (t, 2H, H_i), 1.67 (m, 4H, H_h and H_f), 1.46 (m, 2H, H_g), 1.43 (s, 9H, Boc-H).



APpic2Btn (**5**), assignment of ¹H-NMR signals.

Small letters designate hydrogen atoms in synthesis products **(3)** and **(5)**, for use with the ¹H-NMR peak lists given above.

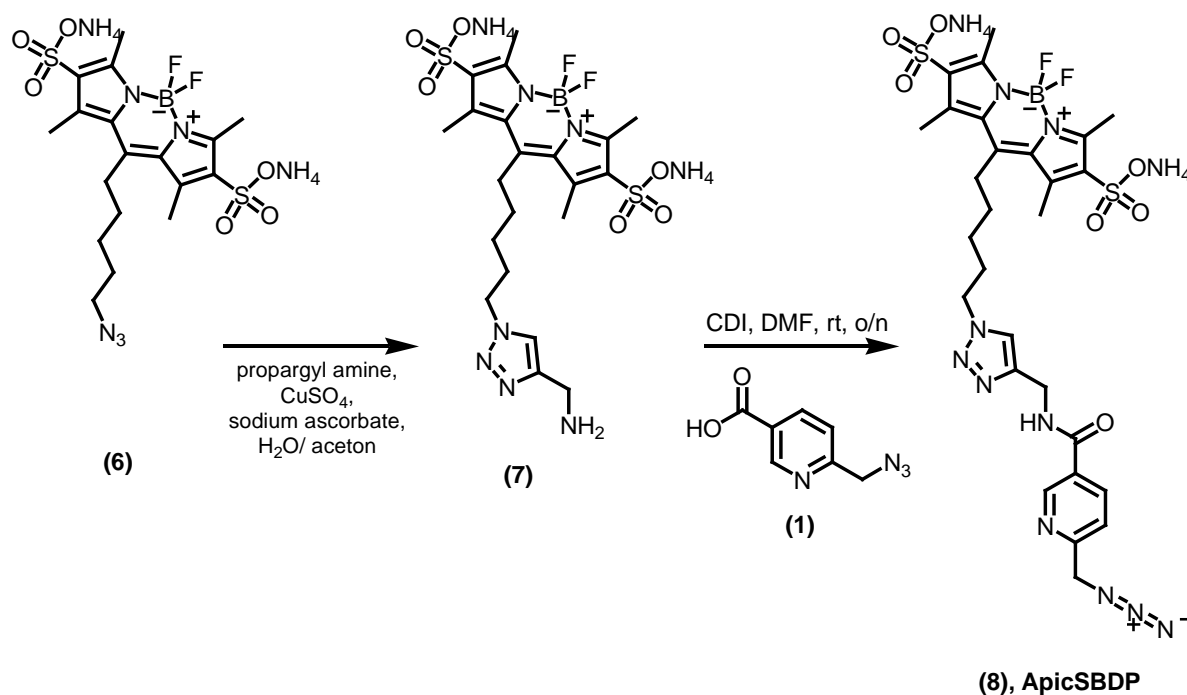
Deprotection and coupling to **(1)**: The mono-protected linker **(3)** was dissolved in 500 μl dry DCM. 450 μl trifluoro acetic acid (TFA) was added and the mixture stirred until deprotection to **(4)** was completed (monitored by TLC, 1 h). Solvent and excess TFA were evaporated. Compound **(1)** (68 mg, 0.38 mmol, 1 eq) was dissolved in 1 ml DMF. CDI (62 mg, 0.38 mmol, 1 eq) was added under Ar atmosphere and the mixture stirred for 4 h. The deprotected linker **(4)** (142 mg, 0.38 mmol, 1 eq), dissolved in DMF (1 ml) and triethylamine (105 μl, 0.76 mmol, 2 eq), was slowly added to the activated carboxylic acid and the reaction mix stirred over night. The solvents were evaporated and the residue purified over a silica gel column with CHCl₃/MeOH 3/1 to yield 80 mg APpic2Btn (**5**) as a colorless semisolid (0.15 mmol, 40%, $R_f = 0.67$).

¹H-NMR (400 MHz, CDCl₃, for atom labels see scheme above): 9.06 (m, 1H, H_p), 8.22 (dd, 1H, H_q), 7.84 (m, 1H, H_r), 4.50 (s, 2H, H_s), 4.47 (m, 1H, H_b), 4.28 (m, 1H, H_a), [3.7-3.6 (m, 8H) and 3.54 (t, 2H) and 3.38 (m, 2H): 12H, H_j, H_k, H_l, H_m, H_n, H_o], 3.10 (m, 1H, H_e), 2.9 (m, 1H, H_c), 2.73 (m, 1H, H_d), 2.17 (t, 2H, H_i), 1.62 (m, 4H, H_h and H_f), 1.40 (m, 2H, H_g).

MS (ESI +): 557.3 ([M+Na]⁺, calculated: 557.227), 535.3 ([M+H]⁺, calculated: 535.245).

ApicSBDP

ASBDP (8-(5-azidopentyl)-4,4-difluor-1,3,5,7-tetramethyl-4-bora-3a,4a-s-indacene-2,6-disulfonic acid disodium salt, **(6)**) was synthesized as described before [3] and coupled to propargylamine:



Synthesis of ApicSBDP.

In a plastic reaction tube, 22 mg **(6)** (42 μ mol, 1 eq), sodium ascorbate (14.1 μ mol, 0.34 eq), and CuSO₄ (7.1 μ mol, 0.17 eq) were dissolved in 4 ml water and 3 ml acetone. Propargylamine (6.93 mg, 126 μ mol, in 300 μ l ethanol) was added and the mixture incubated at -20°C for 24 h, under occasional shaking. The reaction was quenched with saturated aqueous ammonium chloride solution (1 ml) at rt. The solvents were removed partially and the residue purified on a preparative scale column (Macherey-Nagel Polygoprep C60-50 C₁₈) with a gradient of 0-70% methanol.

Yield: 15 mg orange-red solid, containing 85% product **(7)** (R_f = 0.65 in H₂O/MeOH 7/3, RP-TLC) and 15% educt (R_f = 0.5).

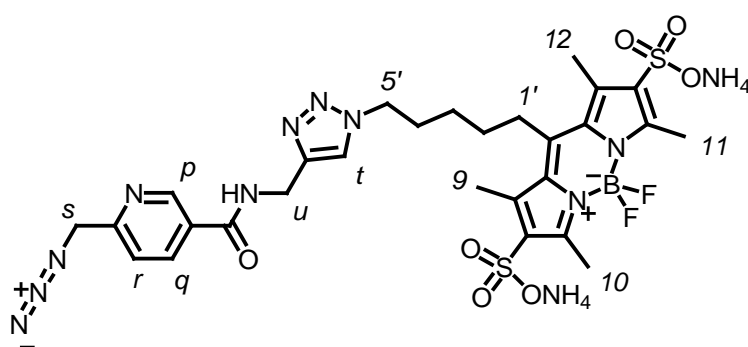
¹H-NMR (D₂O, 400 MHz, for atom labels see scheme below): 1.3-1.6 (m, 4H, 2'-CH₂, 3'-CH₂), 1.97 (m, 2H, 4'-CH₂), 2.64+2.67+2.70+2.74 (4x s, 12H, 9'-, 10'-, 11', 12'-CH₃), 3.00 (m, 2H, 1'-CH₂), 4.33 (s, 2H, H_u), 4.45 (t, 2H, 5'-CH₂), 8.09 (s, 1H, H_t).

Coupling of 6-azidomethylnicotinic acid (**(1)**, synthesized as described in [1]) to **(7)**:

(1) (3.56 mg, 20 μ mol, 1 eq) was dissolved in 1 ml DMF under Ar. CDI (3.24 mg, 20 μ mol, 1 eq) in 100 μ l DMF was added and the mixture stirred at rt for 4 h. To this was added a solution of **(7)** (20 μ mol, 1 eq) in 1 ml DMF and the mixture stirred at rt for 16 h. The solvent was removed in vacuo and the crude product purified twice on HPLC (0-70% methanol gradient) to yield 7 mg of ApicSBDP **(8)** as a red semi-solid (9.1 μ mol, 45%).

MS, ESI(-): $m/z = 366.1$ (calculated: $m/z [M - (2x NH_4)]^{2-} = 366.094$).

1H -NMR (D_2O , 400 MHz, for atom labels see scheme below): 1.3-1.6 (m, 4H, 2'-CH₂, 3'-CH₂), 1.93 (m, 2H, 4'-CH₂), 2.55+2.62+2.65+2.66 (4x s, 12H, 9'-, 10'-, 11', 12'-CH₃), 2.99 (m, 2H, 1'-CH₂), 4.35 (s, 2H, H_u), 4.45 (t, 2H, 5'-CH₂), 4.63 (s, 2H, H_s), 7.38 (m, 1H, H_r), 8.03 (m, 2H, H_q and H_t), 8.75 (m, 1H, H_p).



ApicSBDP **(8)**, assignment of 1H -NMR signals.

Small letters designate hydrogen atoms in synthesis products **(7)** and **(8)**, for use with the 1H -NMR peak lists given above.

Supplementary References

1. Uttamapinant, C., A. Tangpeerachaikul, S. Grecian, S. Clarke, U. Singh, P. Slade, K. R. Gee, and A. Y. Ting. 2012. Fast, cellcompatible click chemistry with copper chelating azides for biomolecular labeling. *Angew. Chem. Int. Ed.* **124**: 5954-5958.
2. Favre, A., J. Grugier, A. Brans, B. Joris, and J. Marchand-Brynaert. 2012. 6- Aminopenicillanic acid (6-APA) derivatives equipped with anchoring arms. *Tetrahedron* **68**: 10818-10826.
3. Hofmann, K., C. Thiele, H. Schott, A. Gaebler, M. Schoene, Y. Kiver, S. Friedrichs, D. Lutjohann, and L. Kuerschner. 2014. A novel alkyne cholesterol to trace cellular cholesterol metabolism and localization. *J. Lipid Res.* **55**: 583-591.

Troubleshooting guide for the labeling of alkyne lipids with azide reporters for lipid imaging

Problem 1: Alkyne lipid signal is low (in the range of control samples not supplemented with alkyne lipids).

- Increase sensitivity by using a picolyl-containing azide reporter.
- Increase sensitivity by increasing the reporter concentration.
- Increase sensitivity by increasing the catalyst concentration.
- Increase alkyne lipid concentration or prolong alkyne lipid supplementation.

Problem 2: After considering the suggestions in Problem 1, the alkyne lipid signal is still low (in the range of control samples not supplemented with alkyne lipid).

- Verify the cellular uptake of alkyne lipid (by extracting cellular lipids, click labeling and lipid analysis by thin layer chromatography). Note that while some lipids are hydrophilic enough to be dissolved in serum/BSA containing cell culture media, some more hydrophobic lipids such as ceramides or sterols need to be provided bound to BSA, cyclodextrin or in liposomes.
- Freshly prepare the CuTfB catalyst solution every time. CuTfB undergoes oxidation/disproportionation to Cu(II) (blue) in humid/aqueous environments.
- Verify that the click reaction mix is free of chloride ions and not acidic. The presence of detergents such as saponin during the click reaction decreases click sensitivity, hence wash the samples at least once with click buffer before performing the click reaction.

Problem 3: The azide reporter is producing too much background fluorescence.

- Use a lower concentration of azide reporter.
- Increase the number of washing steps. Increase the duration of the washes for example to washing overnight.
- Use a different azide reporter.

Problem 4: Cells are unevenly stained.

- Mix the click reaction mix thoroughly before addition to the sample.
- Provide the reporter in excess to the alkyne lipid amount in the sample.
- Ensure complete immersion over the whole area of the sample by using a sufficient volume of click reaction mix.