## Supporting Information

# The synthesis of AX7593, a quinazoline-derived photoaffinity probe for EGFR

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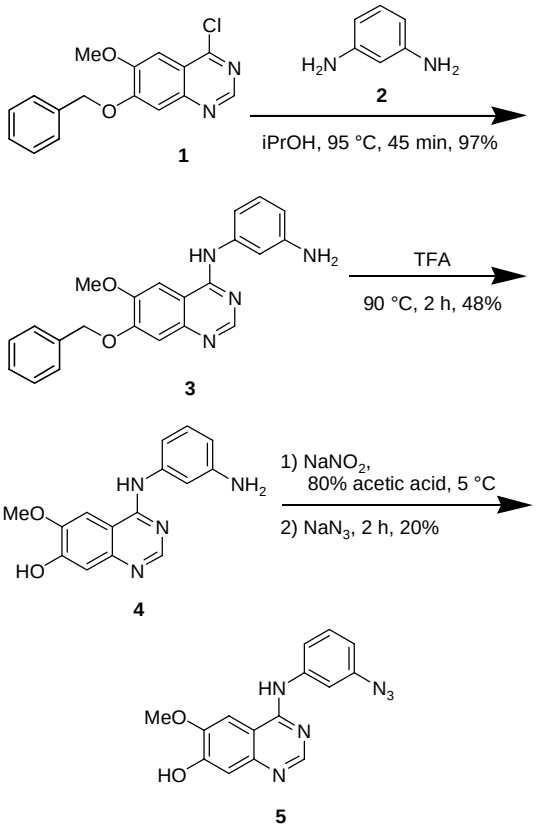
**Experimental Procedures and Data**

**Materials and Methods.** All solvents and reagents were obtained from the Sigma-Aldrich Chemical Company (Milwaukee, WI) unless otherwise indicated; the mixed 5- and 6-succinimidyl esters of tetramethylrhodamine (TAMRA-OSu) were obtained from Molecular Probes (Eugene, OR). [$\gamma$-$^{32}$P]-ATP was purchased from Amersham Biosciences (Piscataway, New Jersey). EGFR, purified from A431 cells, was obtained from Dr. Paul Bertics, University of Wisconsin Medical School (Madison, WI). Ecolite scintillation cocktail was purchased from ICN Biomedicals (Costa Mesa, CA). Other kinases and kinase inhibitors were obtained from Calbiochem (now EMD Biosciences; La Jolla, CA). Proton nuclear magnetic resonance ($^1$H NMR) spectra were recorded on a Bruker 400 MHz NMR spectrometer using the residual peaks in the deuterated solvents as internal standards. Samples were dissolved in deuterated dimethylsulfoxide ($d_6$-DMSO) unless otherwise indicated. Preparative HPLC was carried out on a reverse phase Polaris $C_{18}$ column (5 µ column; 150 mm × 21 mm; Varian; Torrance, CA) using a binary system of water and acetonitrile with 0.1% trifluoroacetic acid (TFA) as a modifier. Analytical LC/MS samples were carried out on a Polaris $C_{18}$ column (5 µ column; 50 × 4.6 mm; Varian; Torrance, CA) using a binary system of water and acetonitrile with 0.1% TFA as a modifier. HPLC purity was determined using the LC/MS trace at 220 and/or 254 nm.

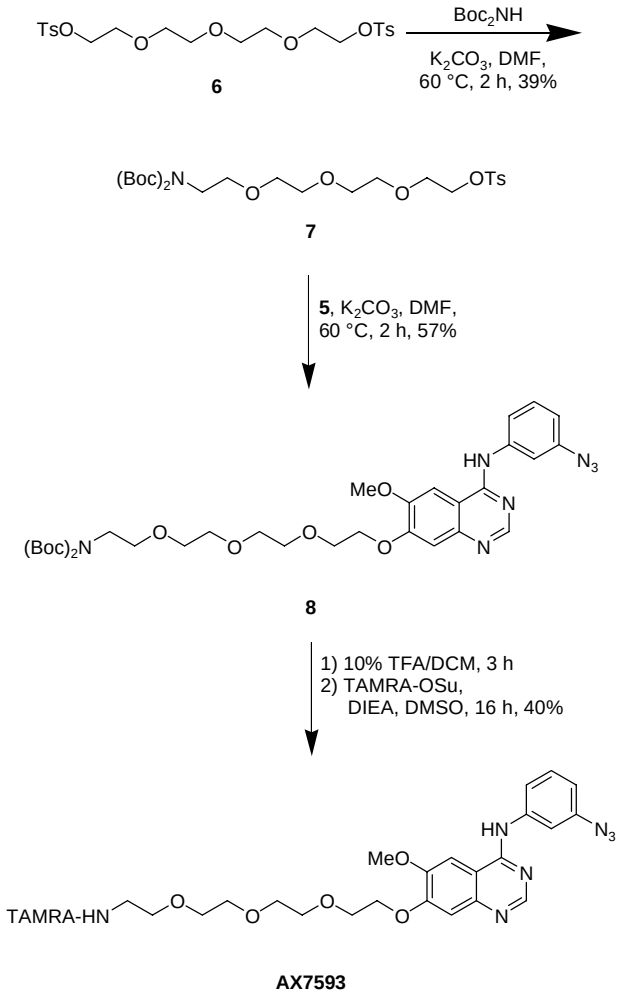



**N-(7-Benzyloxy-6-methyoxy-quinazolin-4-yl)-benzene-1,3-diamine** (**3**)**.** A solution of 7-benzyloxy-4-chloro-6-methoxy-quinazoline (compound **1**, 350 mg, 1.16 mmol) and 1,3-phenylenediamine (compound **2**, 377 mg, 3.49 mmol) in iPrOH (10.0 mL) was heated at reflux in a sealed tube at 95 $^{o}$C for

45 minutes. The solution was allowed to cool to room temperature and the precipitate was collected using vacuum filtration. The filtered solid was dried under high vacuum, yielding compound **3** as a dark green-blue solid (410 mg, 97% yield). $^1$H-NMR δ: 10.70 (bs, 1H), 8.69 (s, 1H), 8.11 (s, 1H), 7.51 (d, 2H, *J* = 4.0 Hz), 7.41 (m, 5H), 7.11 (t, 1H, *J* = 8.0 Hz), 6.91 (s, 1H), 6.84 (d, 1H, *J* = 8.0 Hz), 6.54 (d, 1H, *J* = 8.0 Hz), 6.41 (s, 1H), 5.31 (s, 2H), 3.97 (s, 3H). ESMS: 373.1 $[M + H]^+$.

**4-(3-Amino-phenylamino)-6-methoxy-quinazolin-7-ol** (**4**)**.** A solution of compound **3** (326 mg, 0.875 mmol) in trifluoroacetic acid (10.0 mL) was heated at reflux in a sealed tube for 2 hours at 90 $^oC$. The acid was removed under vacuum and the solid was washed with ether, yielding a black solid. The solid was dissolved in a minimal amount of DMSO and purified by reverse phase chromatography on a $C_8$ column. The fractions containing the desired product in >97% purity by HPLC were collected and lyophilized overnight affording compound **4** as a tan solid (160 mg, 48% yield). $^1$H-NMR δ: 10.83 (s, 1H), 8.74 (s, 1H), 8.03 (s, 1H), 7.16 (m, 2H), 6.86 (bs, 2H), 6.62 (m, 1H), 3.96 (s, 3H). ESMS: 283.1 $[M + H]^+$.

**4-(3-Azido-phenylamino)-6-methoxy-quinazolin-7-ol** (**5**)**.** To a solution of compound **4** (141 mg, 0.50 mmol) in 80% acetic acid (5.0 mL) cooled in an ice bath was added sodium nitrite (38 mg, 0.55 mmol). After 5 minutes, sodium azide (36 mg, 0.55 mmol) was added and the reaction was stirred for 2 hours. The solvent was removed under vacuum and the resulting solid was dissolved in a minimal amount of DMSO and purified on a $C_{18}$ reverse phase chromatography column. Multiple fractions containing the desired product were collected but only those containing the desired product in >97% purity by HPLC were collected and lyophilized overnight affording compound **5** as a pale yellow powder (31 mg, 20% yield). $^1$H-NMR δ: 10.84 (bs, 1H), 8.77 (s, 1H), 8.01 (s, 1H), 7.49 (m, 3H), 7.18 (s, 1H), 7.07 (m, 1H), 3.98 (s, 3H). ESMS: 281.1 $[M + H - 28]^+$, 309.1 $[M + H]^+$.




**Toluene-4-sulfonic acid 2-{2-[2-(2-*N*,*N*-bis-*tert*-butoxycarbonylamino-ethoxy)-ethoxy]-ethoxy}-ethyl ester** (**7**)**.** A solution of di-*tert*-butyl iminodicarboxylate (9.51 g, 43.8 mmol), di(ethylene glycol) di-*p*-tosylate (compound **6**, 20.0 g, 39.8 mmol), and $K_2CO_3$ (5.50 g, 39.8 mmol) in anhydrous DMF (10.0 mL) was heated at 60 $^oC$. After 2 hours, EtOAc (50 mL) and $H_2O$ (30 mL) were added. The organic layer was washed with 1 N HCl (2 × 20 mL), $H_2O$ (2 × 15 mL), and brine (2 × 15 mL). The organic layer was dried over $Mg_2SO_4$ and concentrated to dryness. The solid was redissolved in a minimal amount of $CH_2Cl_2$, loaded onto a silica gel column, and eluted using a gradient of 10-30% EtOAc/Hexanes. The product was dried via rotary evaporation, affording compound **7** as a colorless oil (8.50 g, 39% yield). $^1$H-NMR δ ($CD_3CN$): 7.79 (d, 2H, $J$ = 8.4 Hz), 7.44 (d, 2H, $J$ = 8.0 Hz), 4.11 (m, 2H), 3.71 (t, 2H, $J$ = 6.0 Hz), 3.60 (m, 2H), 3.52 (m, 6H), 3.48 (s, 4H), 2.45 (s, 3H), 1.47 (s, 18H). ESMS: 348.1 $[M + H - 200]^+$, 570.2 $[M + Na]^+$.

**{2-[2-(2-{2-[4-(3-Azido-phenylamino)-6-methoxy-quinazolin-7-yloxy]-ethoxy}-ethoxy)-ethoxy]-ethyl}-dicarbamic acid *tert*-butyl ester** (**8**)**.** A solution of compound **7** (39 mg, 0.07 mmol) in DMF

(1.0 mL) was added dropwise to a stirred suspension of compound **5** (20 mg, 0.06 mmol) and $K_2CO_3$ (18 mg, 0.13 mmol) in anhydrous DMF (1.0 mL) under $N_2$ gas at ambient temperature. The reaction was heated to 60 $^oC$ for 16 hours. The cooled solution was filtered through a syringe filter (0.45 μm) and purified on a $C_{18}$ reverse phase chromatography column. The fractions containing the desired product in >97% purity by HPLC were collected and lyophilized overnight affording compound **8** as a yellow hydroscopic solid (25 mg, 57% yield). $^1$H-NMR δ: 10.64 (bs, 1H), 8.77 (s, 1H), 8.00 (s, 1H), 7.51 (m, 3H), 7.24 (s, 1H), 7.04 (d, 1H, *J* = 6.8 Hz), 4.30 (m, 2H), 3.98 (s, 3H), 3.83 (m, 2H) 3.53 (m, 12H), 1.41 (s, 18H). ESMS: 684.2 $[M + H]^+$, 706.2 $[M + Na]^+$.

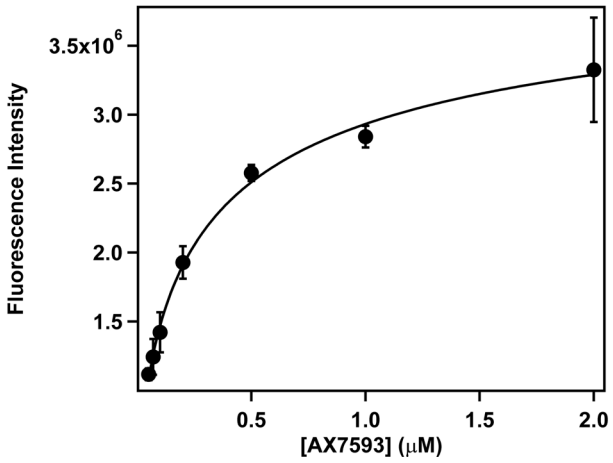
**AX7593.** To a stirred solution of compound **8** (16 mg, 0.02 mmol) in $CH_2Cl_2$ (1.0 mL) was added trifluoroacetic acid (100 μL) and the reaction was stirred at ambient temperature for 3 hours. The solvent was removed using a steady stream of $N_2$ gas without heating, affording a yellow oil. A solution of the yellow oil in anhydrous DMSO (0.5 mL) was added dropwise to a stirring solution of TAMRA-OSu (12 mg, 0.02 mmol) and diisopropylethylamine (8 μL) in anhydrous DMSO (0.5 mL). The reaction was stirred for 16 hours at ambient temperature, and then purified on a $C_{18}$ reverse phase chromatography column. The fractions containing the desired product in >97% purity by HPLC were collected and lyophilized overnight affording compound **AX7593** as a red powder (8 mg, 40% yield). $^1$H-NMR δ ($CD_3CN$): 8.46 (d, 2H, *J* = 20.8 Hz), 8.11 (d, 1H, *J* = 7.6 Hz), 7.56 (m, 4H), 7.32 (m, 3H), 6.93 (d, 2H, *J* = 9.6 Hz), 6.85 (m, 1H), 6.68 (d, 2H, *J* = 9.2 Hz), 6.53 (s, 2H), 4.29 (bs, 2H), 3.88 (bs, 5H), 3.66 (m, 12H), 3.15 (s, 12H). ESMS: 448.7 $[M + 2H]^{2+}$, 896.2 $[M + H]^+$. MALDI-FTMS (DHB): m/z 896.3717 ($C_{48}H_{49}N_9O_9$+H requires 896.3726).

**Determination of EGFR $K_b$ value for AX7593.** Reactions (20 μL) contained 0.5 pmol EGFR (immunoaffinity purified EGFR obtained from Dr. Paul Bertics, University of Wisconsin, Madison, WI) in 50 mM HEPES, 150 mM NaCl, 0.03% Triton X-100 pH 7.4. Photoaffinity labeling reactions were carried out in duplicate with varying amounts of probe (0.05 – 2 μM) at ambient temperature in a polypropylene 96-well plate. The reaction plate was irradiated at 254 nm for 1 minute at approximately 3000 μW/cm$^2$ using a Stratalinker 1800 (Stratagene, La Jolla, CA). After irradiation was complete, each reaction was mixed with 20 μL 2 × Laemmli sample buffer and heated to 95 °C for 2 minutes. Samples were then loaded onto 8 × 10 cm, 12.5% Laemmli gels and electrophoresed at 200V. The gels were scanned on a flatbed scanner (FMBio II, Hitachi Genetic Systems; Alameda, CA) with excitation provided by the 532-nm line of a 50 mW neodymium-doped yttrium-aluminum-garnet laser. A 605 nm bandpass filter was used to detect TAMRA fluorescence. The scanned images were analyzed using Image Analysis 3.0.0.21 software (Hitachi Genetic Systems; Alameda, CA) and the integrated band intensities (normalized for volume) were calculated for the labeled proteins. For each inhibitor, fluorescence intensity was plotted versus the inhibitor concentration, and the points were fit iteratively to the following equation to estimate the $K_b$ of the inhibitors:

$$F = F_{\min} + \frac{(F_{\max} - F_{\min})}{\{[1 + (\frac{K_b}{[probe]})^s\}}$$

where $F$ is the fluorescence intensity, $F_{\min}$ is the fluorescence intensity in the absence of probe, $F_{\max}$ is the fluorescence intensity of EGFR when saturated with the probe, and $s$ is a parameter determining the slope of the curve. Curve-fitting was carried out using the Igor Wave Metrics software (Wave Metrics; Lake Oswego, OR).

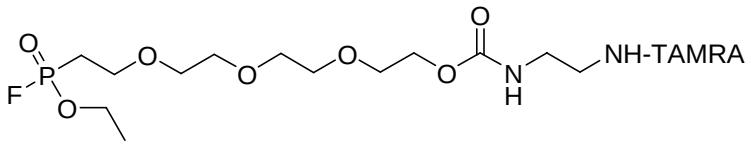
**Supplementary Figure 1.** Plot of observed TAMRA fluorescence from photolabeled EGFR versus concentration of **AX7593**. Post photolabeling of EGFR, samples were separated from unbound probe using SDS-PAGE, visualized with a flatbed laser scanner ($\lambda_{exc}$ = 532 nm), and the emission (605 nm bandpass filter) measured using gel analysis software. The curves drawn are the best fit to an unconstrained four parameter Hill function and yielded a $K_b$ value for **AX7593** of 280 ± 13 nM.




**$IC_{50}$ determination of AX7593 for EGFR.** The $IC_{50}$ of **AX7593** for EGFR was determined by quantitation of the incorporation of $^{32}P_i$ from [$\gamma$-$^{32}$P]-ATP into the peptide substrate poly(Glu-Ala-Tyr) in the presence of varying amounts of **AX7593**. EGFR (3 μg/mL) was mixed with poly(Glu-Ala-Tyr) (1.5 mg/mL) in 50 mM HEPES, pH 7.4, in the presence of varying amounts of **AX7593**. [$\gamma$-$^{32}$P]-ATP was mixed into a solution containing ATP (30 μM), $MgCl_2$ (15 mM), and $MnCl_2$ (6 mM) in 50 mM HEPES, pH 7.4. The reaction was initiated by mixing the EGFR solution (20 μL) with the ATP solution (10 μL). The final concentrations of **AX7593** ranged from 0.01 μM to 10 μM. After a 30 minute incubation at ambient temperature, the reaction was quenched with 30 μL of cold 1% phosphoric acid. 45 μL of the 60 μL reaction was spotted onto Whatman p81 filter paper and the filter paper was washed with 0.5% phosphoric acid (3 × 5 minutes). The filter paper was dried, added to a scintillation vial containing scintillation cocktail (5 mL), and counted on a Beckman-Coulter LS6500 Scintillation Counter.

**Determination of AX7593 photoaffinity labeling efficiency.** The integrated fluorescence of TAMRA was correlated to probe-labeled protein concentration by use of a calibration curve generated from treatment of known quantities of trypsin with a TAMRA-fluorophosphonate serine hydrolase probe (**9**). Fluorophosphonate probe labeling of serine hydrolases is fast and under the conditions of the labeling

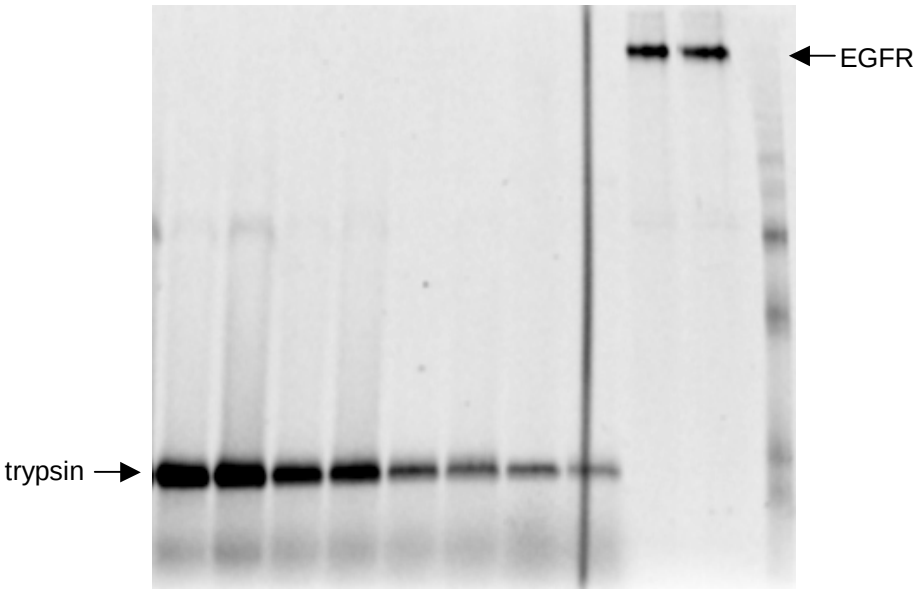
experiment, saturation of the active site (i.e. quantitative labeling) occurs.[1] Trypsin (0.048-0.375 pmol) in 50 mM HEPES, 100 mM NaCl, pH 7.4 was treated with the TAMRA-fluorophosphonate probe **9** (2 μM) in a total reaction volume of 20 μL and was allowed to stand at ambient temperature for 30 minutes. Likewise, EGFR (0.375 pmol) was incubated with **AX7593** (2 μM) in 50 mM HEPES, 100 mM NaCl, 0.03% Triton X-100, pH 7.4 for 30 minutes then was irradiated at 254 nm for 1 minute at approximately 3000 μW/cm$^2$ using a Stratalinker 1800 (Stratagene; La Jolla, CA). Further incubation or irradiation, respectively, for the TAMRA-fluorophosphonate probe **9** or **AX7593** did not result in further labeling. Each reaction was mixed with 20 μL 2× Laemmli sample buffer and heated to 95 °C for 2 minutes. Samples were then loaded onto 8 × 10 cm, 12.5% Laemmli gels and electrophoresed at 200V. The gels were scanned on a flatbed scanner (FMBio II, Hitachi Genetic Systems; Alameda, CA) with excitation provided by the 532-nm line of a 50 mW neodymium-doped yttrium-aluminum-garnet laser. A 605 nm bandpass filter was used to detect TAMRA fluorescence. The scanned images were analyzed using Image Analysis 3.0.0.21 software (Hitachi Genetic Systems; Alameda, CA) and the integrated band intensities (normalized for volume and corrected for background) were calculated for the labeled proteins.



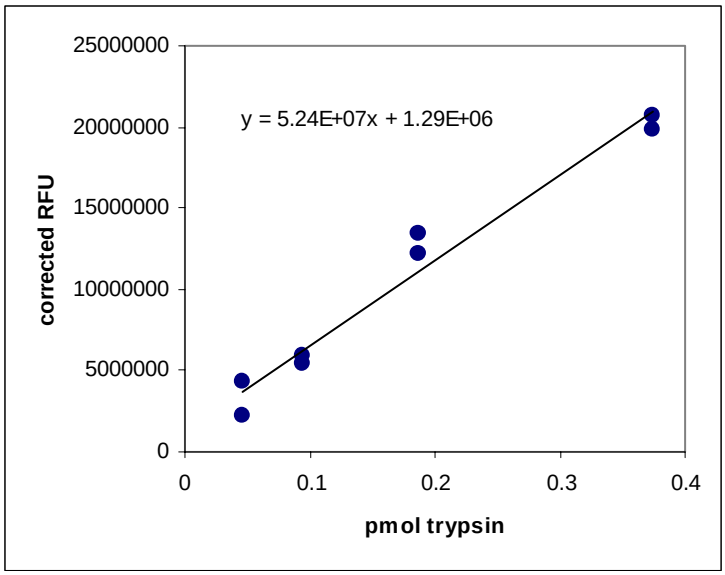

**9**
TAMRA-fluorophosphonate serine hydrolase probe

[1] Patricelli, M. P., Giang, D.K., Stamp, L. M., Burbaum J. J. (2001) Direct visualization of serine hydrolase activities in complex proteomes using fluorescent active site-directed probes. *Proteomics 1*, 1067-1071.

A)


EGFR
trypsin

B)


y = 5.24E+07x + 1.29E+06
corrected RFU
pmol trypsin
25000000
20000000
15000000
10000000
5000000
0
0
0.1
0.2
0.3
0.4

C)

| pmol EGFR labeled | Corrected RFU |
|---|---|
| 0.375 | 6011047 |
| 0.375 | 6637346 |

[**AX7593**-EGFR complex] (x) solved for using:

$K_b$ = [EGFR free][**AX7593** free]/ [**AX7593**-EGFR complex] = ([EGFR]-x)([**AX7593**]-x)/ x

and $K_b$ = 280e-9, [EGFR] = 0.375 pmol/20 μL = 1.875e-8 M, [**AX7593**] = 2 μM

% EGFR bound = [**AX7593**-EGFR complex]/ [EGFR total]

= **88%**

Average corrected RFU for 0.375 pmol of labeled EGFR: **6.3e6**

Expected corrected RFU for 0.330 pmol (0.375 pmol × 0.88) bound EGFR based on calibration curve: **1.86e7**

% of expected corrected RFU: **34%**