ORGANIC LETTERS

2004
Vol. 6, No. 21
3715−3718

Synthesis of AX7593, a Quinazoline-Derived Photoaffinity Probe for EGFR

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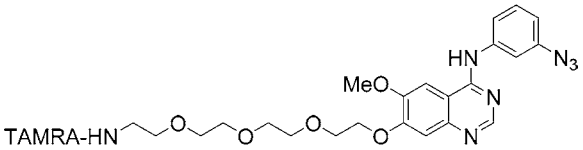
Received July 13, 2004

ABSTRACT



AX7593

The synthesis of a photoaffinity probe for EGFR is described. O-Alkylation of 4-(*meta*-azidoanilino)-6-methoxy-7-hydroxy-quinazoline with a protected tetraethyleneglycol linker followed by the attachment of tetramethylrhodamine yielded the fluorescent probe AX7593. Photoaffinity labeling of EGFR by AX7593 (K_b = 280 nM) was shown to have an efficiency of 34% and to be competitive with the EGFR inhibitors PP2 and AG1478.

Photoaffinity reagents are useful biochemical tools for investigating protein identification, structure, localization, and active-site determination.[1] Three structural components are important in the design of such probes: a protein recognition element that incorporates a photoreactive group, a linker, and a tag useful for detection and quantitation (e.g., a fluorophore). Unlike a probe that incorporates an affinity label,[2] the association of a photoaffinity probe with its target is reversible until photoactivated. After irradiation, the initially inert photoreactive group is converted into a highly reactive electrophilic species that forms a covalent bond with the protein to which it is bound. For example, photoexcitation of aryl azides yields a reactive aryl nitrene capable of insertion into amino acid residues with both nucleophilic and nonnucleophilic side chains.[3]

As part of our efforts to design fluorescent probes to study signal transduction processes,[4] we present herein the synthesis of a fluorophore-tagged photoaffinity probe for the epidermal growth factor receptor (EGFR). EGFR is a transmembrane receptor and a member of the tyrosine kinase family that has been found to be abnormally activated in a variety of solid tumors.[5] This activation of EGFR results in autophosphorylation, which drives signal transduction pathways leading to tumor growth. A number of small-molecule inhibitors that interfere with aberrant EGFR family signaling are currently in preclinical development or in clinical trials for this therapeutically important kinase.[6]

The preparation of the (*meta*-azidoanilino)-quinazoline **5** (Scheme 1) began with the S_NAr displacement of the chloride group of 7-benzyloxy-4-chloro-6-methoxy-quinazoline (**1**)[7] with *meta*-phenylenediamine (**2**) in isopropyl alcohol heated

(1) (a) Fedan, J. S.; Hogaboom, G. K.; O'Donnell, J. P. *Biochem. Pharmacol.* **1984**, *33*, 1167−1180. (b) Tometsko, A. M.; Richards, F. M., Eds. *Ann. N. Y. Acad. Sci.* **1980**, *346*, 134−502.

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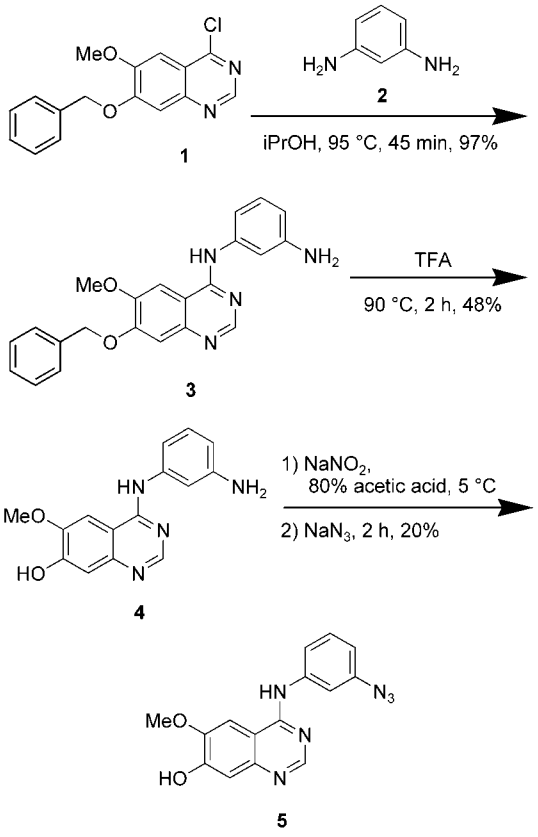
(4) Shreder, K. R.; Liu, Y.; Nomanhboy, T.; Fuller, S. R.; Wong, M. S.; Gai, W. Z.; Wu, J.; Leventhal, P. S.; Lill, J. R.; Corral, S. *Bioconjugate Chem.* **2004**, *15*, 790−798.

(5) Vlahovic, G.; Crawford, J. *Oncologist* **2003**, *8* (6), 531−538.

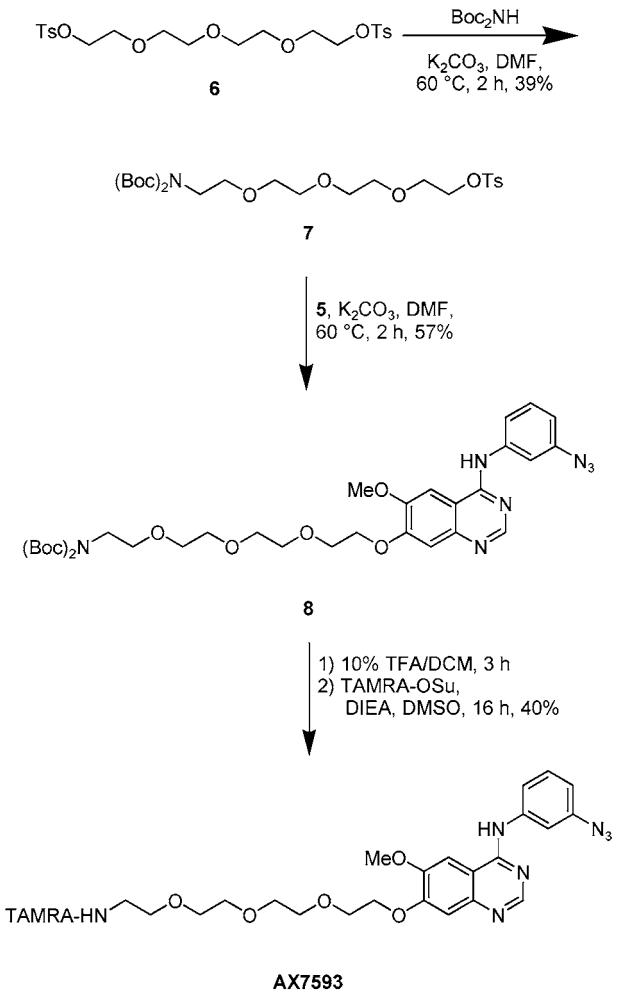
(6) Janmaat, M. L.; Giaccone, G. *Oncologist* **2003**, *8* (6), 576−586.

10.1021/ol048656a CCC: $27.50
Published on Web 09/23/2004

Scheme 1. Synthesis of the (*meta*-Azidoanilino)-quinazoline **5**



Scheme 2. Synthesis of **AX7593**



at reflux, which yielded the anilino-quinazoline **3** in 97% yield.[8] Deprotection of the *O*-benzyl group to give compound **4** was achieved in trifluoroacetic acid (TFA) heated at reflux. Treatment of this intermediate with sodium nitrite in 80% acetic acid produced a yellow diazonium salt that was not isolated. In situ treatment of this intermediate with sodium azide generated the aryl azide **5** with the expected concomitant evolution of N_2 gas.

Once this aryl azide-containing precursor was in hand, attention was turned to the attachment of the linker and fluorophore (Scheme 2). Displacement of one of the tosylate groups of tetraethylene glycol di-*p*-tosylate (**6**) using the potassium salt of di-*tert*-butyl iminodicarboxylate (Carpino's acid-labile phthalimide equivalent)[9] yielded the protected amino tetraethyleneglycol linker **7** in 39% yield. O-Alkylation of the (*meta*-azidoanilino)-quinazoline **5** with compound **7** was achieved using K_2CO_3 in DMF to give the intermediate **8**. Deprotection of the *N*,*N*-di-Boc group was accomplished quantitatively in 10% TFA/dichloromethane. Higher concentrations of TFA resulted in the decomposition of the aryl azide to the corresponding arylamine. **AX7593** was produced by the treatment of this ammonium salt with the mixed succinimidyl esters of 5-(and 6)-carboxytetramethylrhodamine (TAMRA-OSu) and diisopropylethylamine (DIEA) in DMSO. TAMRA has previously been shown to be a superior fluorescent tag for fluorophosphonate-based serine hydrolase probes.[10] Purification of **AX7593** was achieved using C_{18} reversed-phase HPLC (0.1% TFA as a modifier) followed by lyophilization. Use of this purification scheme eliminated any significant trace of degradants in the final product. When the HPLC eluant was removed using rotary evaporation, the increased concentration of the less volatile TFA resulted in product decomposition.

To test whether this compound would label EGFR, the title kinase was incubated with **AX7593** over a range of concentrations (0.01−2 μM) for 30 min.[11] After this period

(7) Compound **1** was synthesized from the commercially available 4-benzyloxy-3-methoxy-vanillin in seven steps. See: (a) Julia, M.; Manoury, P.; Voillaume M. C. *Bull. Soc. Chim. Fr.* **1965**, *5*, 1417−1423. (b) Althius, T. H.; Hess, H.-J. *J. Med. Chem.* **1977**, *20*, 146−149. (c) Hennequin, L. F.; Thomas, A. P.; Johnstone, C.; Stokes, E. S. E.; Plé, P. A.; Lohmann, J.-J. M.; Ogilvie, D. J.; Dukes, M.; Wedge, S. R.; Curwen, J. O.; Kendrew, J.; Lambert-van der Brempt, C. *J. Med. Chem.* **1999**, *42*, 2, 5369−5389.

(8) Certain meta-substituted anilino-quinazolines have been shown to be more potent inhibitors of EGFR when compared to ortho- and para-substituted congeners; see: Rewcastle, G. W.; Denny, W. A.; Bridges, A. J.; Zhou, H.; Cody, D. R.; McMichael, A.; Fry, D. W. *J. Med. Chem.* **1995**, *38*, 3482−3487.

(9) Carpino, L. A. *J. Org. Chem.* **1964**, *29*, 2820−2824.

(10) (a) Patricelli, M. P.; Giang, D. K.; Stamp, L. M.; Burbaum, J. J. *Proteomics* **2001**, *1*, 1067−1071. (b) Adam, G. C.; Burbaum, J.; Kozarich, J. W.; Patricelli, M. P.; Cravatt, B. F. *J. Am. Chem. Soc.* **2004**, *126* (4), 1363−1368.

(11) All photoaffinity labeling experiments were conducted in 50 mM HEPES, 150 mM NaCl, 0.03% Triton X-100, pH 7.4.

of equilibration, the samples were illuminated with 254 nm light to photoactivate **AX7593** and tag any complexed enzyme. Unreacted probe was separated from EGFR using SDS-PAGE, and the fluorescent signal of TAMRA tagged EGFR was measured using a flatbed gel scanner. Because the degree of photoaffinity labeling is directly proportional to the amount of probe bound prior to irradiation, the K_b (binding constant) value could be calculated and was determined to be 280 ± 13 nM. As a control experiment, **AX7593** (1 μM) was photoactivated in the presence of Akt1, MAPKAP kinase 2, and Cdk2, and no labeling of these kinases was observed.

The ability of the probe to inhibit the EGFR-catalyzed phosphorylation of poly(Glu-Ala-Tyr) with [γ-^{32}P]-ATP was also examined.[12] EGFR activity, as measured by the incorporation of $^{32}P_i$ into the peptide substrate, was plotted versus the concentration of **AX7593** (0.01 μM to 10 μM, Figure 1). When the resultant curve was fit to a four-parameter Hill

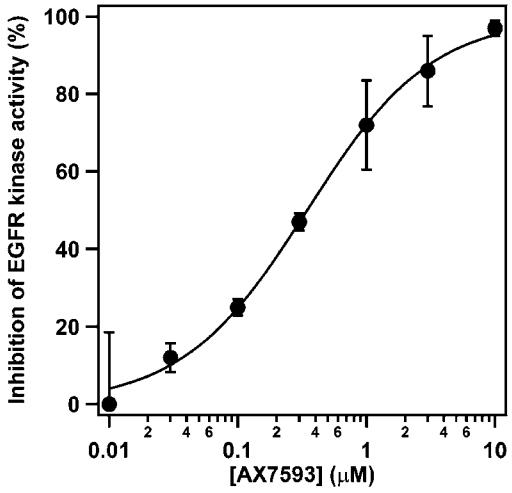


Figure 1. Inhibition of EGFR kinase activity by **AX7593**. The curve drawn is the best fit of the data to a four-parameter Hill function, which yielded an IC_{50} value of 350 ± 20 nM.

function, the IC_{50} of **AX7593** for EGFR was calculated to be 350 ± 20 nM, a value in good agreement with the binding constant determined by photoaffinity labeling (vide supra).

To determine the labeling efficiency of **AX7593** (i.e., the percentage of probe-complexed enzyme that is covalently tagged), a calibration curve was generated from the labeling of trypsin with a TAMRA-containing serine hydrolase fluorophosphonate probe. Use of this calibration curve allowed the amount of TAMRA fluorescence visualized using SDS-PAGE to be correlated to the absolute quantity of labeled enzyme. When EGFR was labeled by **AX7593**, it was determined that the amount of TAMRA fluorescence observed was 66% less than expected for the complete labeling of **AX7593**-bound EGFR. Thus, the labeling efficiency of

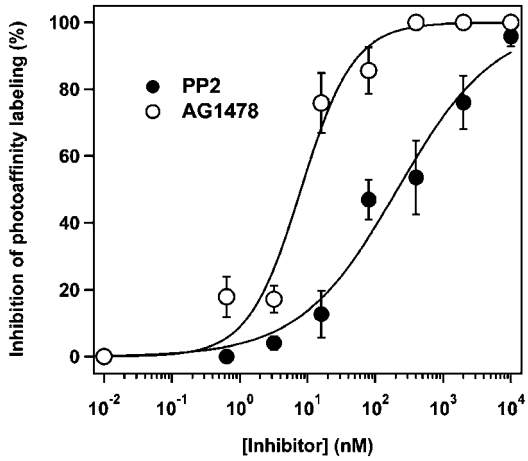


Figure 2. Inhibition of **AX7593** labeling of EGFR by PP2 (●) and AG1478 (○) at various concentrations. The curves drawn are the best fit of the data to a four-parameter Hill function, which yielded IC_{50} values of 210 ± 53 nM and 8.0 ± 1.8 nM for PP2 and AG1478, respectively.

AX7593 was assigned as 34%. The labeling efficiencies of aryl azide-containing photoaffinity probes are known to vary widely from single digit percentages to near quantitative values.[13]

Next, labeling experiments in the presence of EGFR inhibitors were performed to determine if **AX7593** could be used to assay for small-molecule inhibition (Figure 2).[14] Two known EGFR inhibitors, PP2 (IC_{50} = 480 nM)[15] and AG1478 (IC_{50} = 7 nM),[16] were used as model compounds. After incubation of EGFR (10 nM) with varying concentrations of both inhibitors (0.5−10 000 nM), **AX7593** (2 μM) was added and the probe was photoactivated at 254 nm. When the percent inhibition of photoaffinity labeling was plotted as a function of inhibitor concentration and the resulting curves were fitted to a four-parameter Hill function, the calculated IC_{50} values for PP2 (IC_{50} = 210 ± 53 nM) and AG1478 (IC_{50} = 8.0 ± 1.8 nM) were in close agreement with literature values.

In summary, we developed a route for the synthesis of **AX7593**, a quinazoline-based photoaffinity probe for EGFR. Importantly, small-molecule competition of **AX7593** photolabeling was shown to be a useful method of extracting EGFR IC_{50} values. Such inhibition of photoaffinity labeling has the potential to be adapted to a nonradioactive, high-throughput screening method of new compounds as inhibitors of EGFR. The linear nature of the synthesis described herein

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(13) Bayley, H.; Knowles, J. R. *Methods Enzymol.* **1977**, *46*, 69−114.

(14) TAMRA-tagged activity-based probes have been shown to be useful in the high-throughput screening of small molecule inhibitors directly in proteomes. See: (a) Nomanbhoy, T. K.; Rosenblum, J.; Aban, A.; Burbaum, J. J. *Assay Drug Dev. Technol.* **2003**, *1* (2), 137−146. (b) Leung, D.; Hardouin, C.; Boger, D. L.; Cravatt, B. F. *Nat. Biotechnol.* **2003** *21* (6), 687−691.

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(16) (a) Levitzki, A.; Gazit, A. *Science* **1995**, *267*, 1782−1788. (b) Osherov, N.; Levitzki, A. *Eur. J. Biochem.* **1994**, *225*, 1047−1053.

allows for the ready incorporation of other kinds of linkers and tags. Additional studies with **AX7593** and related photoaffinity probes for EGFR will be reported in due course.

Acknowledgment. The authors thank Drs. John Kozarich, Katrin Szardenings, and David Campbell for helpful suggestions concerning this work or manuscript.

Supporting Information Available: Synthetic experimentals for compounds **3**−**5**, **7**−**8**, and **AX7593**; EGFR K_b and IC_{50} determination for **AX7593**; determination of **AX7593** labeling efficiency. This material is available free of charge via the Internet at http://pubs.acs.org.

OL048656A