Synthesis of a Photoreactive AMPAR Antagonist: ANQX

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Background

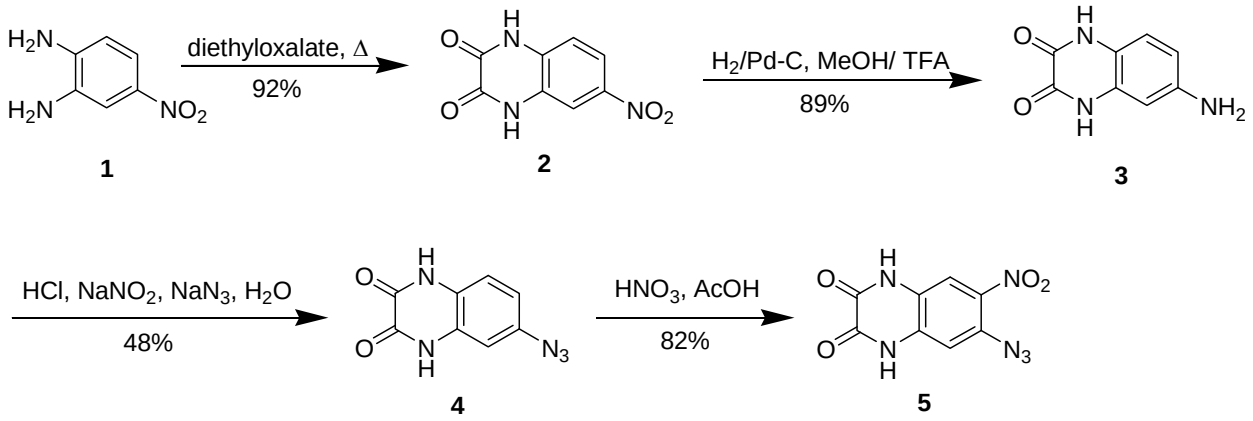
AMPARs are transmembrane ionotropic glutamate receptors which mediate the majority of fast excitatory synaptic transmission in the brain, and are believed to be involved in the processes of mammalian learning and memory.[1] It is believed that the regulation of the number of AMPARs present at synapses of neurons is a critical mechanism underlying memory and learning. There is therefore an interest in developing AMPAR antagonists to study trafficking at these receptors to and from the neuronal cell surface. AMPARs are located on cell surfaces and internally, so it was necessary to prepare an antagonist which was membrane impermeable in order to selectively target AMPARs present only at the cell surface. ANQX (6-Azido-7-nitro-1,4-dihydroquinoxaline-2,3-dione) is a photoreactive AMPAR antagonist which can insert covalently into proteins to which it is initially non-covalently bound. This instertion permanently inhibits the AMPAR. Since ANQX is a membrane impermeable compound, it will only inhibit AMPARs located on the surface of neuronal cells. Upon photoactivation of ANQX by UV light, surface AMPARs will become inhibited, allowing detection of trafficking of fresh non-inhibited AMPARs from inside the cell to the synaptic membrane. The new AMPARs are then able to bind glutamate, their endogenous agonist. This approach thereby enables a method for monitoring both the rate and the quantitative level of the trafficking of AMPARs from inside cells to neuronal surfaces.

Chemistry

The synthesis of ANQX is shown below (Scheme 1). Most of the steps involved in the synthetic sequence were fairly straightforward and gave an adequate yield of product, the catalytic hydrogenation provided some difficulties. In order to obtain the reduction product (**3**), we

decided to use 6-Amino-1,4-dihydroquinoxaline-2,3-dione (**2**) dissolved in methanol with the addition of a 14% v/v of trifouroacetic acid instead of a 95:5 ethyl acetate:ethanol mixture as described in the original synthesis.[1] The modification was developed because we were unable to reproduce the results described in the original synthesis, as in our hands, the reagent did not dissolve, nor subsequently react. After the amine (**3**) was formed, it was transformed to an azide using sodium azide and sodium nitrite. We also made a deviation from the literature in this step by washing the precipitated product with a 1:1 mixture of cold ethanol and water, rather than neat ethanol, followed by 2N hydrochloric acid. This change enabled us to obtain a better yield of the intermediate, as too much was lost in the filtrate when using the original method. Finally, nitration of the azide (**4**) allowed us to obtain the target compound, ANQX (**5**) with 89.5% yield. It should be noted that a problem that revealed itself during the synthesis was the extremely poor solubility of compounds **(3)**, **(4)** and **(5)**. All of these compounds proved only slightly soluble in a large concentration of DMSO, which limited available techniques that might have been used for purification. The final ANQX product was obtained at a 92% purity level as estimated by proton NMR; the melting point was observed to be 300.5-301.5 ºC in comparison to the melting point of 301-302 ºC which was described in the literature.

Scheme 1. Synthesis of ANQX



Experimental

General. Thin-layer chromatography (TLC) analyses were carried out on Analtech (Newark, DE) silica gel GHLF 0.25 mm plates using 254nm UV detection. Melting points were determined in open glass capillaries on an Electrothermal MEL-TEMP 3.0 apparatus. Mass

spectrometry was completed using an Agilent 1200 Series Mass Spectrometer. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Varian spectrometer at 400 MHz and 100 MHz respectively. For ^{1}H-NMR, tetramethylsilane (TMS) was used as internal standard. All starting materials and reagents were commercially available from Sigma-Aldrich and were used without further purification.

6-Nitro-1,4-dihydroquinoxaline-2,3-dione (2). 4-Nitrophenylenediamine (25.0 g, 163 mmol) was suspended in diethyl oxalate (100 mL) and heated to reflux under an Argon atmosphere. After 24 hours, the mixture was allowed to cool to room temperature and the precipitate collected via filtration. The precipitate was washed with EtOH to afford 31.21 g (92%) of an orange solid **(2)**, mp>315 ºC. It was used in the next step without further purification. ^{1}H-NMR (400 MHz, (d^{6}-DMSO): δ 7.23 (d,1H, Ar*H*); 7.94 (m, 2H, Ar*H*); 12.16 (s,1H, N*H*); 12.36 (s,1H, N*H*)

6-Amino-1,4-dihydroquinoxaline-2,3-dione (3). A solution of **(2)** (4.0 g, 17.0 mmol), MeOH (200 mL) and trifluoroacetic acid (28 mL) were added to a 500 mL Parr flask containing Pd/C (10% dry on charcoal, 0.3 g), and the reaction vessel purged with argon. The flask was then pressurized to 30 psi with H_2 and shaken for approximately 2 hours using a Parr hydrogenation apparatus. Afterwards, the solution was filtered through Celite and washed with MeOH. The resulting filtrate was rotary evaporated to afford **(3)** 3.02 g (89.5%) as a brownish solid; mp>315 ºC. This product was used directly in the next step. ^{1}H-NMR (400 MHz, (d^{6}-DMSO): δ 6.84 (dd,1H, Ar*H*), 6.89 (d,1H, Ar*H*), 7.08 (d,1H, Ar*H*), 11.93 (d, 2H, 2 N*H*)

6-Azido-1,4-dihydroquinoxaline-2,3-dione (4). To a solution of **(3)** (1.9 g, 10.7 mmol) dissolved in 2N HCl (101.0 mL) at 0 ^{o}C was slowly added a solution of $NaNO_2$ (0.766 g, 11.1 mmol) in H_2O (21.45 mL). The solution was covered in aluminum foil and allowed to stir for 15 minutes. To this reaction mixture NaN_3 (0.705 g, 10.8 mmol) in H_2O (39.84 mL) was added dropwise. The reaction was allowed to warm to ambient temperature, and after stirring for ~10 hours it was cooled to 0 ºC. The precipitate was collected via filtration and washed with 500 mL cold EtOH:H_2O 1:1, followed by 2N HCl. This afforded (**4**) 0.94 g (47.7%) as an orange-tan solid that was used in the next step without further purification; mp:196-197 ºC. ^{1}H-NMR (400

MHz, (d^6-DMSO): δ 6.83 (d, 1H, Ar*H*), 6.88 (dd, 1H, Ar*H*), 7.14(d, 1H, Ar*H*), 11.90 (bs, 1H,N*H*), 11.95 (bs, 1H, N*H*)

6-Azido-7-nitro-1,4-dihydroquinoxaline-2,3-dione (ANQX). To a solution of (**4**) (0.82 g, 4.0 mmol) in AcOH (41.0 mL) was added dropwise a solution of fuming HNO_3 (6.5 mL, 154.6 mmol). After allowing the reaction to stir for ~16 hours, the precipitate was collected via filtration and washed extensively with AcOH, 2N HCl, H_2O, and EtOH. This afforded the final product (**5**), as a light yellow solid (0.815 g, 82.3%); mp 300.5-301.5 ºC. [lit. mp. 301-302 ºC]. ^{1}H-NMR (400 MHz, (DMSO): δ 7.098 (s, 1H, Ar*H*), 7.78 (s, 1H, Ar*H*), 12.098 (bs, 1H, N*H*), 12.193 (bs, 1H, N*H*). ^{13}C-NMR (100 MHz, (DMSO): δ 107.581, 113.189, 123.705, 130.384, 132.154, 135.062, 155.017, 155.709. APCI-MS: negative mode, *m/z* calculated 248.16 for $C_8H_4N_6O_4$; found: 247.6 for $C_8H_3N_6O_4$ (M-1).

Reference

1. Chambers JJ, Gouda H, Young DM, Kuntz ID, England PM. Photochemically knocking out glutamate receptors in vivo. J Am Chem Soc. 2004 Nov 3;126(43): 13886-7. PMID 15506725