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SYNTHESIS OF A NOVEL THIO-ANTHRAQUINONE DERIVATIVE-BASED TISSUE DYE**

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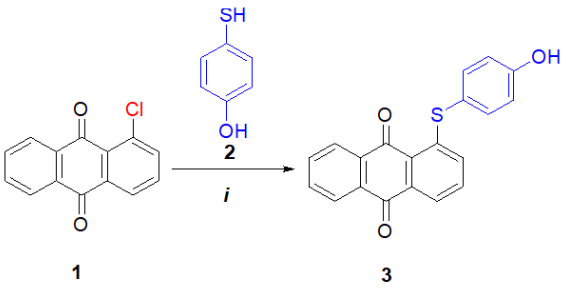
In this study, a new dye, Neurange stain, was developed for histopathological examination of brain tissue using light microscopy. In addition, Neurange dye can be used in routine analyses and microscopic imaging of tissue sections. The synthesis of 1-(4-hydroxyphenylthio)anthracene-9,10-dione (3), a new thio-anthraquinone derivative used in the preparation of Neurange dye, began with the substitution reaction of 4-hydroxythiophenol (2) and 1-chloroanthraquinone (1) as starting materials.[1] Neurange dye shows specific staining of white matter in brain tissue sections. It shows brown-orange staining in areas such as the optic nerve, the cortex in the brain tissue, and areas where axon bundles are located, and especially areas related to the myelin sheath. In conclusion, Neurange dye could be used to distinguish different brain areas using a light microscope.



INTRODUCTION

Anthraquinone molecule is found in nature in St. John's Wort and Aloe Vera plant. Anthraquinones are present in nature and may also be chemically produced in a laboratory.[2,3] Amino- and thio-anthraquinone derivatives can be synthesized by the reaction of anthraquinones with active molecules, nucleophiles. Anti-bacterial, anti-fungal, anti-tumor, anti-HIV, anti-inflammatory, anti-histaminic properties of these derivatives are known.[4–6] Quinones are a class of molecules that can undergo a Faradaic reaction involving two protons and two electrons in an acidic electrolyte and two electrons and cationic species in an alkaline electrolyte.[7–9] Anthraquinones, an important subclass of quinones, exhibit photosensitizing properties by forming singlet oxygen and/or superoxide anion radicals.[10] Anthraquinones, which act as redox-

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active sensitizers under UV irradiation, are defined as a class of redox-active Type I photosensitizers capable of generating $_{O2}$ radicals in basic aqueous media.[11–16] Anthraquinones play an important role in preventing tumor growth and treating the disease through photodynamic therapy-like methods in various types of cancer.[5] The effects of parietin molecule, a natural hydroxy anthraquinone derivative, on breast cancer LM2 cells have been investigated in previous report, and it has been shown that antraquinone derivatives show positive results in the treatment of regional tumors.[5] Alizarin dye, an important hydroxy anthraquinone derivative that is frequently used in tissue dye studies, provides the detection of calcium accumulated in the tissue structure.[17–20] There are several examples of anthraquinone-based tissue dyes in the literature.[21–24] A study by Ortner and colleagues investigated the usability of Deep Red Anthra-quinone 5, a fluorescent dye, in comparison with acridine-orange for digital staining of optically cut skin.[25] Eight fresh frozen-thawed Mohs disard tissue samples were stained with acridine-orange and DRAQ5 and imaged at three wavelengths (488 nm, 638 nm, 785 nm) using an *ex vivo* confocal microscope. The data obtained were evaluated by the investigators for histopathologic characterization. In the evaluation, it was determined that acridine orange stain stained mostly dermal fibers while anthraquinone derivative staining was more specific to the nuclei.

The anthraquinone derivative's rapid staining, nuclear specificity and resistance to photodegradation have been found to support its use as a fluorescent label for digital staining of optically cut skin.[25] The alizarin molecule is an anthraquinone derivative that is biologically active and can be used as a tissue dye.[21–24] The Alizarin red S assay, which is frequently preferred by scientists, is considered the gold standard for osteoblast quantification.[23] In a study by Bernar and colleagues, cell culture experiments with human (SaOs-2) and murine (MC3T3-E1) osteoblasts were performed under increasing concentrations of calcium chloride or calcitonin. After three or four weeks, the mineralization matrix was stained, mixed with alizarin red S and the concentration was measured photometrically.[23] The study results suggested the addition of 2.5 to 10 mM calcium chloride to osteogenic cell culture media to enhance cell differentiation and thus bone matrix mineralization in a time and cost efficient manner, resulting in significantly more sensitive alizarin red S staining.[23]

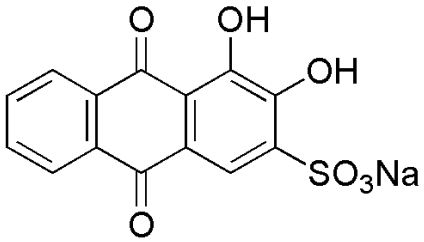


Fig. 1 – Alizarin red S staining.[23]

In a study, mesenchymal stem cells from various teeth and tissues were isolated, and these cells were stained with Alizarin dye, an anthraquinone derivative, during in vitro studies.[26–17] It was stated that the positive alizarin red in the selected groups in the experimental groups indicates the accumulation of calcium in that region.[26] Novel biological dyes have been researched for a variety of uses in recent years.

Biological dyes are widely used as contrast agents or dyes to visualize morphological structures in biological tissues. They are particularly useful in exploratory fields like biology and medicine. Biological stains are applied in various fields such as histology, embryology, microbiology, protein and nucleic acid staining.

Here, Neurange dye, a unique and new biological dye that can visualize some parts of brain tissue under a light microscope, was prepared. Neurange dye can be used in the Chemical, medical, biochemistry, biotechnology, forensic and diagnostic industries. It can also be used for pathological examination of brain samples. Currently, the Golgi staining technique is widely used for light microscopic examination of brain tissues.[27] Golgi's method is a silver staining technique used to visualize nervous tissue under the light microscope. The mechanism of this technique is still largely unknown. So, novel dyes should be developed in order to investigate the histological structures of tissues.

In this study, a new dye, Neurange dye, containing a new compound, 1-(4-hydroxyphenylthio)anthracene-9,10-dione, is reported, which is a first in the literature. In the study, the chemical synthesis procedure of novel compound and the preparation of Neurange dye were discussed. The staining properties of Neurange dye were investigated using rat brain tissue sections.

MATERIALS AND METHODS

Chemicals and instruments

Thin layer chromatography (TLC) was used to observe product formation during the synthesis of the anthraquinone analog in the reflux system and to determine the completion of the reaction. The thin layer chromatography (silica gel $60F_{254}$) DC-plates (aluminum based) were purchased from Merck. The powder silica gel for the column chromatography to purify the novel anthraquinone analogue (3) was obtained from Merck (63–200 µm particle size, 60–230 mesh). Moisture was excluded from the glass apparatus using $CaCl_2$ drying tubes. All chemicals were purchased from Sigma-Aldrich (Taufkirchen,Germany) or Merck (Darmstadt, Germany). The infrared (FT-IR) spectra were recorded using a Tetra JASCO 6600 spectrometer. The ultraviolet–visible spectrawere recorded with a Tetra JASCO V 750 spectrophotom ^{1}HNMR ^{13}C eter. And NMR spectra were recorded on a Varian UNITY INOVA spectrometer at 500 MHz. The chemical shift (ppm) was reported as relative to tetramethylsilane with the solvent resonance ^{1}HNMR and ^{13}CNMR employed as an internal standard. spectra and spectra in $CDCl_3$ refer to the solvent signal center at 7.2 and 77.0 ppm, respectively. Mass spectra were recorded with a LCMS-8030 triple quadrupole spectrometer (Shimadzu, Kyoto, Japan) in ESI (+) polarity. The melting point was measured using a Buchi B-540 apparatus.

Synthesis of 1-(4-hydroxyphenylthio) anthracene-9,10-dione

The novel thio-anthraquinone derivative 1-(4-hydroxyphenylthio)anthracene-9,10-dione **(3)**, was synthesized from the substitution reaction of 1-chloroanthraquinone **(1)** and 4-hydroxythiophenol (2) with the patent method previously developed by the study team (Fig. 1).[1] 1-chloroanthraquinone compound (1 g) **(1)** and 25 mL of ethylene glycol were mixed in the reaction flask, then 4-hydroxythiophenol (0.52 g) **(2)** was added to the reaction flask. A dark yellow reaction mixture was obtained. 10 ml of aqueous potassium hydroxide solution was added to this mixture and the reaction temperature was raised to 110–120 °C. After 48 hours of reflux, the mixture was extracted with chloroform (30 mL) (Fig. 2). During extraction, the water phase was acidified with 5% HCl solution. The organic phase was washed with water and dried with sodium sulfate. The synthesized new analogue **(3)** was purified by column chromatography. The chemical structure of the novel analogue **(3)** was characterized by analytical methods (FT-IR, NMR, MS, UV).

***1- (4-Hydroxyphenylthio)anthracene-9,10-dione* (3).** Orange solid. Mp: 250–251°C. Yield: 0.2 g (14%). Rf (petroleum ether /dichloromethane=1/1): 0.47. FT-IR (ATR) (cm^{-1}): υ = 3438 (OH_{arom}); 3070, 3035 (CH_{arom}); 1554 (C=C); 1655 (C=O). ^{1}H NMR (499.74 MHz, ppm, $CDCl_3$): δ= 5.62 ppm (s, 1H, OH); 6.99–7.01 ppm (d, 2H, J= 7.0 Hz, CH-); 7.13–7.15 ppm (d, 2H, J=7.14 Hz, CH-); 7.46–8.41 ppm (m, 7H, CH_{arom}). ^{13}C NMR (125.66 MHz, ppm, $CDCl_3$): δ= 117.9, 123.97, 126.92, 127.51, 131.72, 132.79, 133.80, 134.40, 137.97, 157.4 ppm (C_{arom} and CH_{arom}); 183.7 ppm (C=O). λmax(logε): 2.02 (283.50 nm), 1.77 (303 nm), 1.07 (433.50 nm). MS [+ESI]: *m/z* = 333.05 $[M+H]^+$, 239.06 $[M-C_6H_5-OH]^+$, $C_{20}H_{12}O_3S$, (M, 332.37 g/mol) (Fig. S1). The molecular weight of synthesized 1-(4-hydroxyphenylthio)anthracene-9,10-dione was 332.37 g/mol.

Preparation of Neurange dye

The thioanthraquinone derivative, 1-(4-hydroxyphenylthio)anthracene-9,10-dione (3) was dissolved in methanol as an organic solvent to prepare the biological tissue stain, called Neurange stain. 10 mg of thioanthraquinone derivative were dissolved in 10 mL of methanol, respectively (in 1 mg/mL organic solvent). Solutions of neurange dye are prepared and carried out at room temperature.

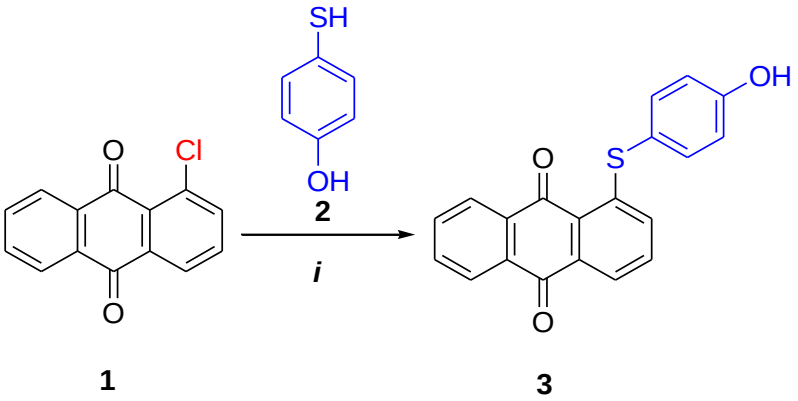
Histological analysis

Rat brain tissue sections are cut at different thicknesses with a cryomicrotome (or microtome and

other instrument) and fixed on a microscope slide. Depending on the sectioning method, pretreatment processes such as passing through alcohol series can be applied before neurange staining. Neurange dye was applied to cover the tissue section. Although the staining time varies, 3 minutes of application may be sufficient. A counterstain, such as hematoxylin, can be applied to improve image quality. After washing, the section was covered with a water-based (or a suitable sealing solution) coverslip and examined microscopically.

RESULTS AND DISCUSSION

The novel 1-(4-hydroxyphenylthio)anthracene-9,10-dione (3) was synthesized according to the conditions of a previous method as shown in Fig. 2.[1]



i: Synthesis of 1-(4-Hydroxyphenylthio)antracene-9,10-dione) 3 (reaction conditions: Ethylene glycol, KOH, 110-120°C)

Fig. 2 – Thioanthraquinone Derivative Reaction Scheme (1-(4-Hydroxyphenylthio)antracene-9,10-dione).

The synthesized substance was purified by column chromatography. The characteristic C = O band for the synthesized 1-(4-hydroxyphenylthio) anthracene-9,10-dione (3) was observed at 1655 cm^{-1} in the FT-IR spectrum. The carbonyl carbon in the anthraquinone skeleton was seen at 183.7 ppm in ^{13}C NMR. The characteristic OH peak was observed at 5.62 ppm in the ^{1}H NMR spectrum. In the mass spectrum of 1-(4-hydroxyphenylthio) anthracene-9,10-dione (3), the molecular ion peak was confirmed as *m/z* = 333.05 $[M+H]^+$. The ion fragment (3) of 1-(4-hydroxyphenylthio) anthracene-9,10-dione was observed at m/z = 239.06 $[M-C_6H_5OH]^+$. In the UV spectrum (MetOH) of the thioanthraquinone molecule, it was observed that the substituent binding to the anthraquinone skeleton shifted the peaks to the visible region. In addition to methanol, the substance was also soluble in organic solvents such as chloroform, dichloromethane, ethylacetate and dimethylsulfoxide (DMSO). Studies have been conducted *in vitro* on various thio and amino anthraquinone derivatives, including this particular structure. A novel thioanthraquinone derivative, 1-(4-hydroxyphenylthio)anthracen-9,10-dione (3), was synthesized within the scope of this study.

Figure 3 shows the absorption spectra of Neurange dye in methanol solution. Three peaks in the region of 185–600 nm were observed for Neurange dye (Fig. 3). The following maximum absorbance values have been observed for peak 1, peak 2, peak 3, respectively; 433.50, 303.00, 283.50.

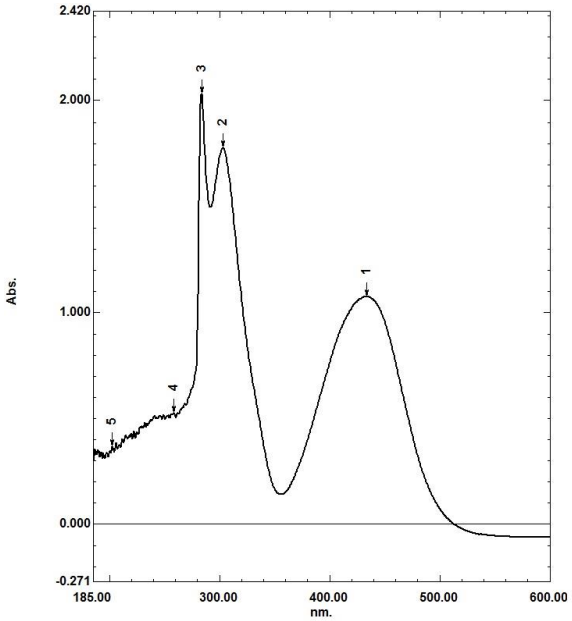


Fig. 3 – UV-Vis spectra of 1-(4-hydroxyphenylthio)anthracene-9,10-dione (3) in a methanol.

Neurange tissue dye shows specific staining properties in the brain tissue mentioned areas.

Figure 4 shows staining pattern of brain section using Neurange dye. The striatum region of the rat brain was used in the staining for comparison. In the striatal area, axon bundles are stained orange (Fig. 4).

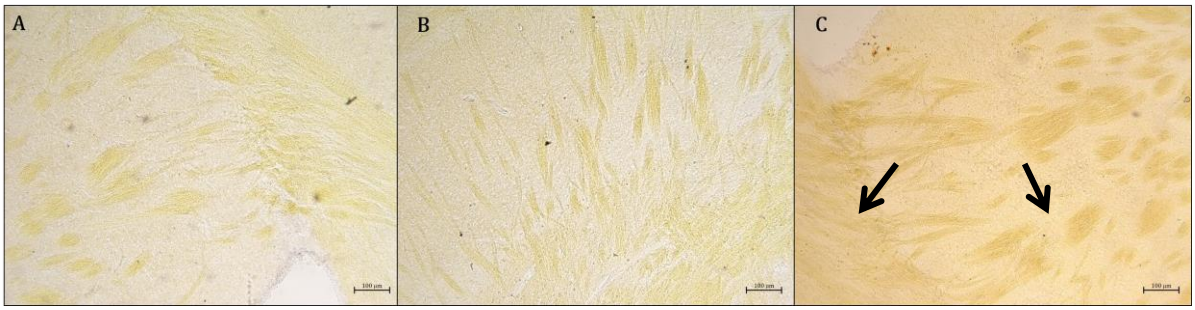


Fig. 4 – Staining pattern of brain tissue section using Neurange dye. Cryosection of formalin-fixed adult rat brain and staining images in the absence of H_2O_2 (A), in the presence of 3% H_2O_2 (B) and 0.05% H_2O_2 after blocking with 3% H_2O_2 solution for 15 minutes (C).

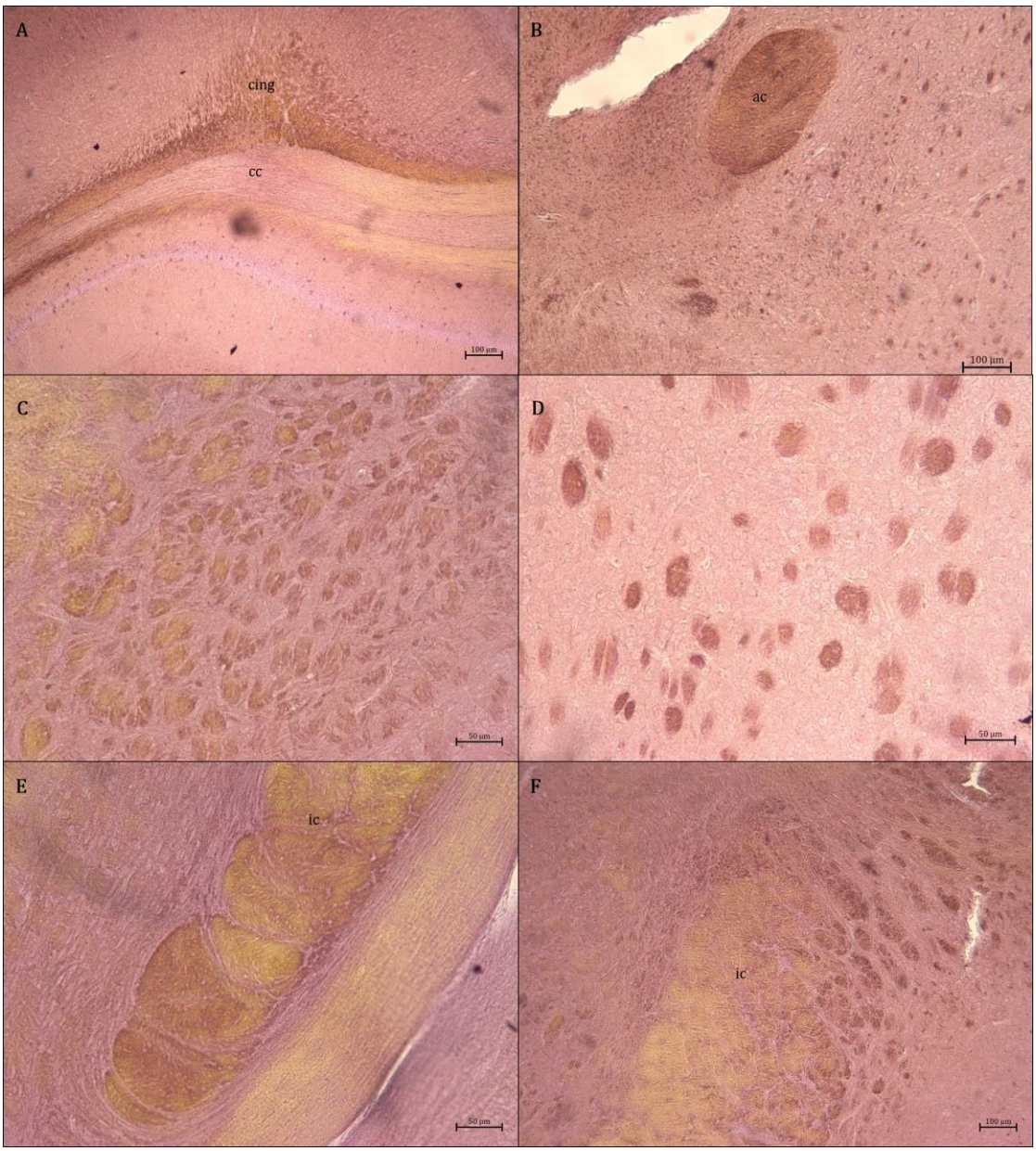


Fig. 5 – Rat brain regions stained with neurange dye. Formalin-fixed adult rat brain was cryosected. Cingulum (cing) and corpus callosum (cc) regions (A), (Anterior commissure) Anterior commission (ac) (B) and Striatum (C) and (D) regions of the brain are stained orange-brown. The interior capsule (ic) regions of the brain can be distinguished (E) and (F).

Figure 5 shows rat brain regions stained with neurange dye. Tissue sections stained with Neurange stain were counterstained with hematoxylin as background. After Neurange staining, the color intensity increases with H_2O_2 concentration. With 3% H_2O_2, axon bundles were stained darker yellow in certain regions (Fig. 5). Especially, cingulum and corpus callosum regions, (Anterior commissure) Anterior commission (ac) and Striatum regions of the brain were stained orange-brown color. The interior capsule (ic) regions of the brain can be distinguished. After Neurange staining, the color intensity increases with H_2O_2 concentration. With 3% H_2O_2, axon bundles were stained darker yellow in certain regions.

The "black reaction", also known as Golgi staining, was initially identified in 1873.[28] Under a microscope, this technique could investigate the neuronal morphology by selectively visualizing the entire architecture of a neuron with a clear background that left thousands of neighboring neurons unstained.[29] The Golgi-Cox staining method is the most widely used because of its convenience and consistently accurate results. However, Golgi staining requires multiple steps in staining procedure and commercial kits are pricey. As a result, alternative methods for light microscopic investigations of brain tissue are required. Neurange dye is quite easy and practical. As a staining profile, it can provide axon-specific staining. Neurange dye is highly sensitive, highly specific, and the concentration detection range is wide, and this range can be expanded by method modification. Neurange dye stains the regions of axon-specific neurofibrils in the rat brain tissue section and allow them to be examined under a light microscope. Antraquinone chromophore groups can be applied as probes in biochemistry, physicochemistry and analytical chemistry owing to their of their strong fluorescence and chromophore structures.[30–32] As a result, White matter-specific dye was developed in mammalian brain tissue axons, which can be distinguished by light microscopy. Previously, it was reported that mammalian brain stained with methylene blue supravital exhibited typical staining patterns in several brain areas showing affinity for the somata and axons of Purkinje cells in the mouse cerebellum.[33] In future, staining characteristics of other mammalian tissues using Neurange dye should be investigated and the staining mechanisms could be investigated in advance.

CONCLUSIONS

The original thioanthraquinone molecule, 1-(4-hydroxyphenylthio)anthracene-9,10-dione (3) synthesized from the reaction of 1-chloroanthraquinone (1) and 4-hydroxy thiophenol (2) was used in the preparation of the Neurange dye for the first time. Although Neurange dye contains the molecule 1-(4-hydroxyphenylthio) anthracene-9,10-dione (3), the dyeing properties of Neurange dye can be improved by adding other molecules or solutions to this dye. In conclusion, Neurange dye could be used in the staining of brain tissues for immediate investigation in a fast manner.

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