Structure Determination and Synthesis of Fluoro Nissl Green: An RNA-Binding Fluorochrome

Craig A. Merlic,*,†,[1a] Soheil Motamed,†,[1b] and Bruce Quinn*,‡

Departments of Chemistry and Biochemistry and Pathology and Laboratory Medicine, University of California, Los Angeles, California 90095-1569

Received October 17, 1994 (Revised Manuscript Received March 14, 1995®)

The isolation, structure determination, and total synthesis of Fluoro Nissl Green, a novel RNA-binding fluorochrome, are reported. Initially obtained in only one part per 10^5, *via* a microwave-induced synthesis from *m*-phenylenediamine, the structure was determined by spectroscopic methods to be 3,8-diamino-10*H*-quindoline. The proposed structure was confirmed by a rational synthesis starting from *m*-phenylenediamine and 2,4-dinitrobenzaldehyde. The key step employed a Friedlander reaction to construct the quindoline ring system from 1-acetyl-6-(acetylamino)-3-indolinone and 2-amino-4-(acetylamino)benzaldehyde. Histochemical studies with synthetic Fluoro Nissl Green demonstrate selective staining of neuronal perikarya and nucleoli.

Introduction

The use of fluorescent organic molecules as optical probes and optical labels has had a major impact on biochemistry, molecular biology, and structural biology. A vast array of fluorochromes are now utilized to selectively stain nucleic acids or protein in analytical methods such as DNA sequencing and to stain components of living or fixed cells for fluorescence-activated cell sorting and fluorescent microscopy.[2] Fluorescent-labeled oligonucleotide probes and antibodies (immunofluorescence) are used to label sequence-specific DNA, mRNA, and proteins.[3] Intercalating agents such as ethidium bromide, proflavin, and acridine orange are used to stain RNA but bind DNA as well. Additionally, a number of fluorescent compounds are employed as counterstains to visualize background cells in neuroanatomic techniques.[4]

In 1989, Quinn reported on the use a novel counterstain, Fluoro Nissl Green (Fluoro-NG), in neuroanatomic studies and in fluorescent immunohistochemical studies. Neuroanatomic studies utilize fluorescent dyes such as rhodamine to label pathways in the central nervous system *in vivo*.[5] Fluorescent counterstains with different emission properties are important because they allow small populations of labeled cells to be visualized in their anatomic context by using multiple filter sets with different band-pass characteristics to visualize the selected probe and the general counterstain. Quinn reported that Fluoro-NG had unique characteristics in staining RNA preferentially compared to DNA *in situ*, as well as excellent resistance to fading, low background staining, low affinity for proteins, and high binding efficiency at neutral pH, characteristics unequaled by any reported fluorochrome counterstain.[4] Further, staining of the perikarya and nucleolus was eliminated by RNAse pretreatment. Although the structural characterization of Fluoro-NG has not been previously determined, the stain has already been utilized in a number of neuroscience research reports using the preparation reported by Quinn.[6]

Since Fluoro-NG has proven to be useful as a fluorescent stain for neuroscience research,[6] its novel characteristics suggest widespread utility in biomedical research and diagnostic applications. For example, the diagnosis of reactive glial changes *versus* low-grade astrocytoma after brain biopsy represents a significant diagnostic challenge for pathologists. In 1987, Sarnat and co-workers proposed that acridine orange staining may be utilized for this diagnosis since neoplastic glial cells display increased cytoplasmic RNA while reactive glial cells do not.[7] However, acridine orange binds both DNA and RNA, whereas Fluoro-NG is selective for RNA in histochemistry and should prove to be a more effective

† Department of Chemistry and Biochemistry.

‡ Department of Pathology and Laboratory Medicine. Present address: Department of Pathology, New York University School of Medicine, 560 First Avenue, New York, NY 10016.

® Abstract published in *Advance ACS Abstracts,* May 1, 1995.

(1) (a) National Science Foundation Young Investigator 1992–1997, Camille Dreyfus Teacher-Scholar 1994–1999. (b) UCLA President's Undergraduate Fellow.

(2) For general methods for staining cell components, see: (a) Ausubel, F. M.; Brent, R.; Kingston, R. E.; Moore, D. D.; Seidman, J. G.; Smith, J. A.; Struhl, K. *Current Protocols in Molecular Biology*; John Wiley & Sons: New York, 1992. (b) Haugland, R. P. *Handbook of Fluorescent Probes and Research Chemicals*; Molecular Probes, Inc.: Eugene, OR, 1992.

(3) For a review of RNA detection by fluorescence, see: Johnson, C. V.; Singer, R. H.; Lawrence, J. W. *Methods Cell Biol.* **1991**, *35*, 73–129.

(4) Schmued, L. C.; Swanson, L. W.; Sawchenko, P. E. *J. Histochem. Cytochem.* **1982**, *30*, 123–128.

(5) (a) Quinn, B.; Weber, E. *Soc. Neurol. Abstr.* **1988**, *14*, 547. (b) Quinn, B. *Soc. Neurol. Abstr.* **1989**, *15*, 309. (c) Quinn, B. Ph.D. Thesis, Stanford University, Stanford, CA, 1989, Chapter 2.

(6) For examples of the use of crude preparations of Fluoro-NG, see: (a) McConnell, S. K.; Ghosh, A.; Shatz, C. J. *Science* **1989**, *245*, 978–982. (b) Alvarez-Buylla, A.; Ling, C.-Y.; Kirn, J. R. *J. Neurosci. Methods* **1990**, *33*, 129–133. (c) Erzurumlu, R. S.; Jhaveri, S. *Dev. Brain Res.* **1990**, *56*, 229–234. (d) von Bartheld, C. S.; Cunningham, D. E.; Rubel, E. W. *J. Histochem. Cytochem.* **1990**, *38*, 725–733. (e) de Lima, A. D.; Voigt, T.; Morrison, J. H. *J. Comp. Neurol.* **1990**, *296*, 159–172. (f) Wicht, H.; Northcutt, R. G. *Brain Behav. Evol.* **1990**, *36*, 315–328. (g) Striedter, G. F. *Brain Behav. Evol.* **1990**, *36*, 355–377. (h) Heredia, M.; Santacana, M.; Valverde, F. *J. Neurosci. Methods* **1991**, *36*, 17–25. (i) Striedte, G. F. *J. Comp. Neurol.* **1991**, *312*, 311–331. (j) Trenga, C. A.; Kunkel, D. D.; Eaton, D. L.; Costa, L. G. *Neurotoxicology* **1991**, *12*, 165–178. (k) Wullimann, M. F.; Meyer, D. L.; Northcutt, R. G. *J. Comp. Neurol.* **1991**, *312*, 415–435. (l) Voigt, T.; de Lima, A. D. *J. Comp. Neurol.* **1991**, *314*, 403–414. (m) Voigt, T.; de Lima, A. D. *J. Comp. Neurol.* **1991**, *314*, 415–428. (n) Ghosh, A.; Shatz, C. J. *J. Neurosci.* **1992**, *12*, 39–55. (o) Gow, A.; Friedrich, V. L.; Lazzarini, R. A. *J. Cell Biol.* **1992**, *119*, 605–616. (p) Striedter, G. F. *Brain Behav. Evol.* **1992**, *39*, 329–357. (q) Santacana, M.; Heredia, M.; Valverde, F. *Dev. Brain Res.* **1992**, *65*, 75–83. (r) Silos-Santiago, I.; Snider, W. D. *J. Comp. Neurol.* **1992**, *325*, 514–526. (s) Voigt, T.; de Lima, A. A.; Beckmann, M. *J. Comp. Neurol.* **1993**, *330*, 48–64. (t) Ghosh, A.; Shatz, C. J. *Development* **1993**, *117*, 1031–1047. (u) McConnell, S. K.; Ghosh, A.; Shatz, C. J. *J. Neurosci.* **1994**, *14*, 1892–1907. (v) Lundell, M. J. *BioTechniques* **1994**, *16*, 434.

(7) Sarnat, H. B.; Curry, B.; Newcastle, N. B.; Trevenen, C. L. *Can. J. Neurol. Sci.* **1987**, *14*, 31–35.

0022-3263/95/1960-3365$09.00/0

discriminator for this differential diagnosis.[5] Widespread application of Fluoro-NG has, however, been hindered by the poor yield from Quinn's original, serendipitously discovered protocol. Furthermore, the unknown molecular structure of Fluoro-NG has impeded the development of a rational synthetic procedure as well as structure–function studies.

Thus, given the potential for further uses in molecular biology and medicine as an RNA probe as well as the interesting structural problem it presented, we initiated a project to elucidate the structure of Fluoro-NG. We report the results of that study and a total synthesis of Fluoro-NG which confirms our structural assignment.

Structure Determination

In order to determine the structure of Fluoro-NG, the initial objective was to obtain spectral data, but this proved to be more difficult than expected due to a very low yield of Fluoro-NG in Quinn's original preparation. Nonetheless, since the preparation proceeded from relatively inexpensive starting materials, enough Fluoro-NG was eventually prepared for characterization. In a typical procedure, *m*-phenylenediamine, glycerol, and acetic acid were combined in water and heated to boiling in a laboratory microwave oven (see Experimental Section). Fluoro-NG was then separated from the lemon yellow solution using reverse phase chromatography on a C_{18} support. Each gram of *m*-phenylenediamine starting material led to substantially less than 100 μg of product, so combined product fractions from six preparative scale reactions, which used 15 g of *m*-phenylenediamine each, were combined and repurified to provide approximately 1.3 mg of Fluoro-NG. Attempts to optimize the yield of this synthesis, through modification of the heating method, reagents, or stoichiometry, were unsuccessful.

Electrospray mass spectrometry of the isolated material indicated a unit mass of 248, and a high-resolution mass spectrum indicated an empirical formula of $C_{15}H_{12}N_4$ (calcd for $C_{15}H_{12}N_4$ 248.1063, found 248.1055). This corresponded to incorporation of two *m*-phenylenediamine molecules and a C_3 fragment into Fluoro-NG. Initial analysis of the proton NMR spectrum was impeded by impurities in the sample; however, two features were diagnostic. First, a unique singlet aryl proton signal at 7.98 ppm suggested a fused polycyclic aromatic ring system. Second, there were two isolated three-proton spin systems indicative of 1,2,4-trisubstituted benzene rings. Each set contained an ortho-coupled doublet signal, an ortho- and meta-coupled doublet of doublets signal, and a meta-coupled doublet signal. A partial ^{13}C NMR was not informative other than to confirm a lack of symmetry.

The presence of a polycyclic aromatic ring system derived from *m*-phenylenediamine and glycerol as a C_3 source suggests a quinoline moiety which could be derived from a Skraup quinoline synthesis.[8] Coupling the spectroscopic data with this chemical proposal leads to four potential structures for Fluoro-NG. They are 3,8-diamino-10*H*-quindoline (**1**), 3,8-diamino-6*H*-quinindoline (**2**), 3,9-diamino-11*H*-indolo[3,2-*c*]quinoline (**3**), and 3,9-diamino-7*H*-indolo[2,3-*c*]quinoline (**4**) (Figure 1). While chemical reactivity considerations of potential reaction

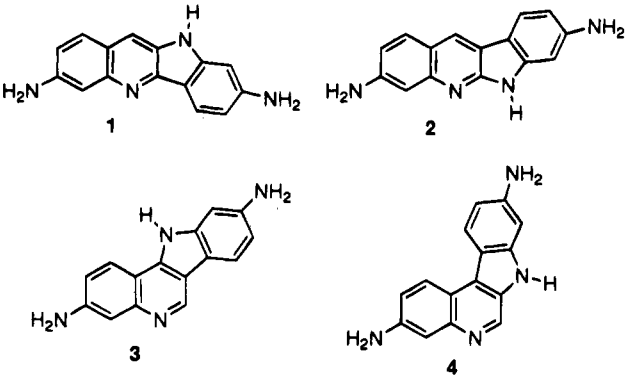


Figure 1. 3,8-Diamino-10*H*-quindoline (**1**), 3,8-diamino-6*H*-quinindoline (**2**), 3,9-diamino-11*H*-indolo[3,2-*c*]quinoline (**3**), and 3,9-diamino-7*H*-indolo[2,3-*c*]quinoline (**4**).

intermediates derived from *m*-phenylenediamine could serve to select the likely structure, further spectroscopic methods were used instead to systematically limit the possible structures. An NOE experiment in methanol-d_4 indicated the proximity of the unique aryl proton with a resonance at 7.92 ppm to a single aromatic proton exhibiting a doublet at 7.70 ppm. This ruled out structures **2** and **4** since NOE enhancements would be expected to show two aryl doublets in structure **2** and to show no other aryl protons in structure **4**. (The H11–H1 and H11–H10 distances in structure **2**, as calculated by MM2, are 2.42 and 2.49 Å, respectively, which suggests potential NOE interactions for both protons.) An additional NOE experiment in dimethyl sulfoxide-d_6 revealed the proximity of a unique exchangeable hydrogen-bonded proton at 10.50 ppm to the unique aryl proton at 7.70 ppm as well as a meta-coupled aryl proton at 6.51 ppm. These results eliminated structure **3** which would lack an NOE interaction between the exchangeable proton and the unique aryl proton. Therefore, the NOE experiments uniquely identified structure **1** as Fluoro-NG. (The H10–H9 and H10–H11 distances in structure **1**, as calculated by MM2, are 2.76 and 2.77 Å, respectively.) Speculation as to whether structure **1** represents the most logical product on the basis of chemical reactivity considerations could arise, but given the extremely low yield of Fluoro-NG from microwave irradiation of *m*-phenylenediamine, the point was considered moot. Instead, this proposal was then confirmed by a rational synthesis of 3,8-diamino-10*H*-quindoline (**1**).

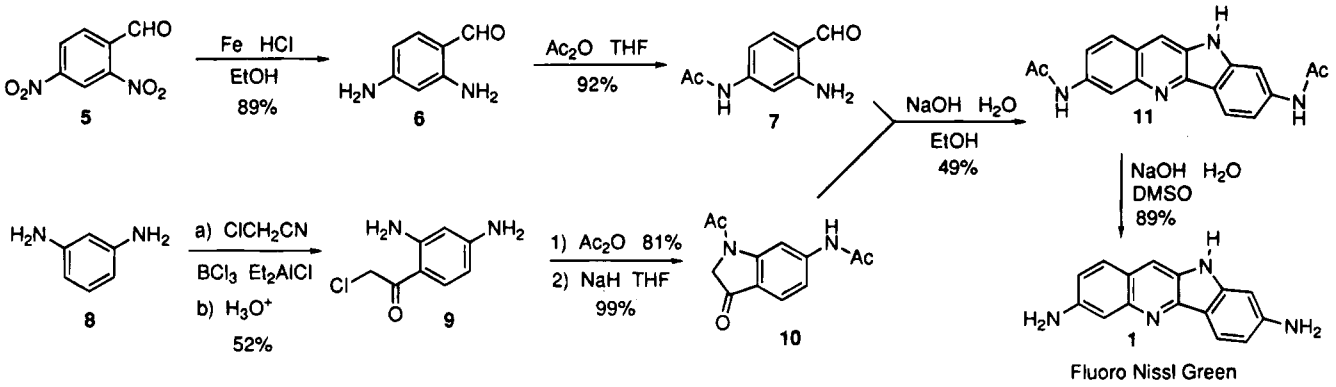
Synthesis

The proposal that 3,8-diamino-10*H*-quindoline (**1**) is indeed the structure of Fluoro-NG was confirmed by an independent synthesis of **1** which is outlined in Scheme 1. The key step employs a Friedlander quinoline synthesis[9] to construct the tetracyclic quindoline ring system from a 3-indolone and a 2-aminobenzaldehyde (**7** + **10** → **11**). In principal, the starting material for each component (**7** and **10**) of the Friedlander reaction could be *m*-phenylenediamine, **8**. Indeed, 3-indolone **10** was prepared from **8** in three steps, but direct formylation of **8** to provide aldehyde **6** through a variety of methods failed; thus, an alternative route from aldehyde **5** was pursued.

(8) For a review on the Skraup quinoline synthesis, see: Manske, R. H. F.; Kulka, M. *Org. React.* (N.Y.) **1953**, *7*, 59–98.

(9) For a review on the Friedlander quinoline synthesis, see: Cheng, C.; Shou-Jen, Y. *Org. React.* (N.Y.) **1982**, *28*, 37–201.

Scheme 1



Commercially available[10] 2,4-dinitrobenzaldehyde (**5**) was reduced with iron in acidic methanol to yield the diamine **6**.[11,12] Other reducing agents such as zinc, tin, and sodium hydrogen sulfide were less effective. Additionally, a selective monoreduction with titanium trichloride to 2-amino-4-nitrobenzaldehyde was unsuccessful.[13] Selective acylation of the diamine **6** at the 4-amino position was predicted on the basis of steric and electronic grounds. Indeed, addition of acetic anhydride to **6** selectively gave the 4-monoacylated aldehyde **7**[14] in 92% yield. Little or no 2-acylated or bisacylated products were obtained in reactions employing 1.5 equiv of acetic anhydride.

The regioselective synthesis of 2-aminophenyl ketones from anilines and nitriles using boron trichloride in the presence of aluminum trichloride, presumably *via* a cyclic transition state involving a boronium cationic species stabilized by a tetrachloroaluminumate anion, has been demonstrated (Sugasawa reaction).[15,16] Thus, a Friedel–Crafts reaction of *m*-phenylenediamine, **8**, with chloroacetonitrile, followed by hydrolysis of the intermediate iminium ion, was used to provide ketone **9**. In our synthesis, however, the reaction was severely hampered by the highly insoluble *m*-phenylenediamine–boron trichloride/aluminum trichloride complex in a wide variety of solvents. Weaker Lewis acids of higher solubility were then substituted for aluminum trichloride despite reports of the optimal activation imparted by the aluminum trichloride.[16] By employing diethylaluminum chloride, reasonable yields of ketone **9** (52%) could be obtained by refluxing in 1,2-dichloroethane with mechanical stirring. In addition to solubility, an additional benefit of diethylaluminum chloride could be its ability to scavenge protons which have been demonstrated to terminate the Sugasawa reaction due to the formation of protonated anilines.[16] Nonetheless, the modest yield of the reaction is most likely a function of the heterogeneous nature of the acylation reaction. Bisacylation of the ketodiamine was readily accomplished with a large excess of acetic anhydride. Subsequent base-promoted ring closure of the bisamide yielded the indolinone **10** in excellent yield.

Coupling of amino aldehyde **7** with indolinone **10** in a base-promoted Friedlander reaction produced the 3,8-bisacylated quindoline **11** in 49% yield.[17] The apparently labile triamide with an acetyl group at the 10 position, initially formed in the cyclization reaction, was not detected. Cyclization reactions employing sodium ethoxide in anhydrous ethanol were not as effective, and use of amine bases in a variety of solvents did not lead to the product. Purification of quindoline **11** was exceedingly difficult as the extremely polar product is only soluble in dimethyl sulfoxide and dimethyl formamide. Limited solubility in methanol allowed for recrystallization using 25:75 methanol/methylene chloride using a large excess of solvent. Successful recrystallization required, however, prior partial purification using reverse phase chromatography on a C_{18} support with aqueous methanol. Deprotection of the amine substituents under acidic conditions provided only limited amounts of product. However, the synthesis was succcessfully completed by deacylation in aqueous sodium hydroxide and purification by normal and reverse phase chromatography to provide Fluoro-NG (**1**) in 89% yield.

The spectroscopic and chemical properties of the rationally synthesized Fluoro-NG (**1**) matched those of the material obtained *via* the microwave synthesis in all respects. Additionally, with quantities of pure Fluoro-NG in hand, further quantitative characterization was possible. Notably, the highly fluorescent product has UV–vis absorptions at 256, 282, 374, 408, and 472 nm. Excitation of Fluoro-NG from 250 to 500 nm leads to intense emission at 510 nm with maximum emission for excitation at 446 nm. Preliminary studies confirmed the complexation selectivity of Fluoro-NG for RNA over duplex DNA and will be reported in a separate communication.

Histochemical Results

The histochemical staining properties of the totally synthetic Fluoro-NG (**1**) matched those of the material obtained *via* Quinn's original procedure.[5] Under blue

(10) 2,4-Dinitrobenzaldehyde is commercially available (Lancaster Synthesis, Inc., Windham, NH) but is moderately expensive. It can be prepared by oxidation of dinitrotoluene with *N,N*-dimethyl-4-nitrosoaniline: Bennet, G. M.; Bell, E. V. *Organic Syntheses*; Wiley: New York, 1943; Collect. Vol. II, p 223–225.

(11) Fox, B. A.; Threlfall, T. L. *Organic Syntheses*; Wiley: New York, 1973; Collect. Vol. V, p 346–351.

(12) Ricci, A. *Ann. Chim. (Rome)* **1958**, *48*, 985–996.

(13) Lehmstedt, K. *Ber. Dtsch. Chem. Ges.* **1938**, *71B*, 808–814.

(14) Compound **7** was previously identified as a photodecomposition product of *sustar* {*N*-[3-[(1,1,1-trifluoromethanesulfonyl)-amino]-4-methylphenyl]acetamide}: Miller, G. C.; Crosby, D. G. *J. Agric. Food Chem.* **1978**, *26*, 1316–1321.

(15) (a) Sugasawa, T.; Toyoda, T.; Adachi, M.; Sasakura, K. *J. Am. Chem. Soc.* **1978**, *100*, 4842–4852. (b) Sugasawa, T.; Adachi, M.; Sasakura, K.; Kitagawa, A. *J. Org. Chem.* **1979**, *44*, 578–586. (c) Adachi, M.; Sasakura, K.; Sugasawa, T. *Chem. Pharm. Bull.* **1985**, *33*, 1826–1835. (d) Sasakura, K.; Terui, Y.; Sugasawa, T. *Chem. Pharm. Bull.* **1985**, *33*, 1836–1842.

(16) Douglas, A. W.; Abramson, N. L.; Houpis, I. N.; Karady, S.; Molina, A.; Xavier, L. C.; Yasuda, N. *Tetrahedron Lett.* **1994**, *35*, 6807–6810.

(17) Zalkow, L. H.; Nabors, J. B.; French, K.; Bisarya, S. C. *J. Chem. Soc. C* **1971**, 3551–3554.

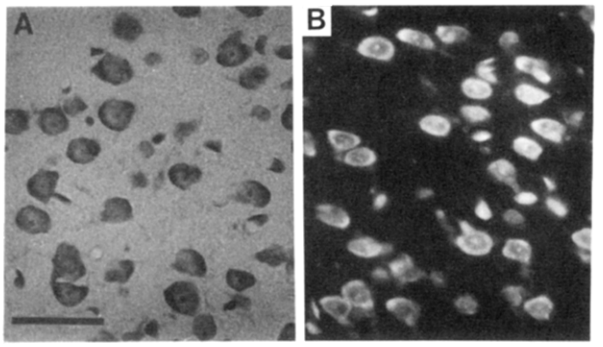


Figure 2. Photomicrographs of rat brain sections showing neurons in the cerebral cortex. Left, conventional Nissl staining with cresyl echt violet. Right, staining by Fluoro-NG visualized with a fluorescein epifluorescence illumination filter. Scale bar: 30 μm.

epifluorescent illumination, brain sections stained with Fluoro-NG displayed bright yellow-green staining of the neuronal perikarya and nucleolus. Background staining was minimal, and the staining pattern matched that of conventional dye Nissl stains used in neuroanatomy (Figure 2). Addition of *p*-phenylenediamine to the glycerol-mounting medium was necessary to prevent fading of the Fluoro-NG stain during illumination. Importantly, RNAse pretreatment of the sample sections eliminated staining of the neuronal perikarya and nucleolus.

Conclusions

Fluoro-NG was isolated from a microwave-promoted synthesis in one part per 10^5, and the structure was determined, by a combination of ^{1}H NMR techniques and mass spectrometry, to be 3,8-diamino-10*H*-quindoline. The structural proposal was confirmed by a rational synthesis starting from *m*-phenylenediamine and 2,4-dinitrobenzaldehyde in five linear steps in a combined yield of 18%.

A number of questions about Fluoro-NG remain unanswered at this point. Current studies are addressing issues such as the mechanism by which microwave irradiation of *m*-phenylenediamine promotes the formation of Fluoro-NG, albeit in low yields; the nature of the interaction between Fluoro-NG and RNA; whether analogs of Fluoro-NG have similar RNA interactions; and the potential uses of Fluoro-NG in biochemical and biomedical research.

Experimental Section

General. All reactions were carried out under nitrogen by standard reaction techniques, unless noted otherwise. Diethyl ether and tetrahydrofuran were distilled from sodium benzophenone ketyl under nitrogen. Methylene chloride and 1,2-dichloroethane were distilled from calcium hydride. *m*-Phenylenediamine was sublimed prior to use. All other reagents were used as received. Flash chromatography was performed on Merck silica gel 60 (230−400 mesh). Reverse phase chromatography was performed using 20 mm, 6 cm^3, C_{18} SepPak cartridge from Waters Corp., Milford, MA.

Microwave Synthesis of Fluoro-NG. Following the procedure of Quinn,[5] freshly sublimed *m*-phenylenediamine (15.0 g), glycerol (15 mL), and acetic acid (7.5 mL) were combined with water (150 mL). The colorless solution was heated in a microwave oven (1600 W, maximum power) for 35 s, swirled, and microwaved an additional 35 s. The lemon yellow solution was diluted with water (1.80 L) and applied to a C_{18} reverse phase column. The product was eluted with 10, 20, and 30% methanolic water solutions (300 mL each). Separation of the RNA selective stain was confirmed by spot lighting since, in aqueous solution, Fluoro-NG exhibits bright green fluorescence under simple flashlight illumination.[18] Strongly fluorescent green product fractions were combined and concentrated *in vacuo* to yield a brown orange residue (3 mg). Combined product fractions from six preparative scale reactions were combined and purified by preparative silica gel TLC (elution with a 70:25:5 $CHCl_3$/MeOH/Et_3N solvent system) to yield 1.3 mg of Fluoro-NG as a brown orange solid (spectral data: *vide infra*).

2,4-Diaminobenzaldehyde (6). Iron powder (3.06 g, 54.8 mmol), water (5 mL), and HCl (100 μL, 12 N) were added consecutively to a solution of 2,4-dinitrobenzaldehyde (**5**) (0.50 g, 2.55 mmol) in ethanol (20 mL). After stirring at 95 °C for 90 min, the reaction mixture was filtered hot. Following an ethanol wash, the filtrates were combined and the solvent was removed *in vacuo*. The crude material was purified by flash chromatography (40:55:5 hexane/ethyl acetate/triethylamine) to yield 0.31 g (89%) of 2,4-diaminobenzaldehyde as yellow crystals. IR (KBr): 3461, 3445, 3322, 3208, 1645, 1626, 1578, 1536, 1445, 1345, 1248, 1217, 1148, 810 cm^{-1}. ^{1}H NMR (360 MHz, acetone-d_6): δ 5.43 (2H, br s), 5.90 (1H, d, J = 2.1 Hz), 6.04 (1H, dd, J = 8.5, 2.1 Hz), 6.70 (2H, br s), 7.15 (1H, d, J = 8.5 Hz), 9.47 (1H, s). ^{13}C NMR (90.6 MHz, acetone-d_6): δ 97.5, 105.5, 112.2, 138.4, 153.9, 155.6, 190.5. MS (relative intensity) (70 eV): 137.07 (25), 136.07 (16), 136.06 (M$^+$, 100), 135.06 (16), 108.07 (39), 107.06 (10), 91.04 (12), 81.00 (12). Mass calcd for $C_7H_8N_2O$ 136.0637, found: 136.0640.

2-Amino-4-(acetylamino)benzaldehyde (7). Freshly distilled acetic anhydride (272 μL, 3.0 mmol) was added to a solution of 2,4-diaminobenzaldehyde (**6**) (0.272 g, 2.0 mmol) in methylene chloride (34 mL). After the mixture was stirred for 48 h at rt, the product was filtered out as a yellow precipitate and washed with hexanes (250 mL). After 30 min, a second crop of precipitate was separated and washed with hexanes. Both batches of precipitate were combined to yield 0.328 g (92%) of 2-amino-4-(acetylamino)benzaldehyde as yellow crystals. Mp: 168 °C. IR (KBr): 3420, 3302, 3184, 2816, 2737, 1686, 1640, 1610, 1572, 1433, 1273, 1207, 1152 cm^{-1}. ^{1}H NMR (400 MHz, acetone-d_6): δ 1.96 (3H, s), 6.61 (1H, dd, J = 8.5, 1.9 Hz), 6.83 (2H, br s), 7.24 (1H, d, J = 1.9 Hz), 7.28 (1H, d, J = 8.5 Hz), 9.18 (1H, br s), 9.57 (1H, s). ^{1}H NMR (360 MHz, DMSO-d_6): δ 2.04 (3H, s), 6.68 (1H, d, J = 8.2 Hz), 7.18 (2H, br s), 7.24 (1H, s), 7.39 (1H, J = 8.2 Hz), 9.62 (1H, s), 10.03 (1H, s). ^{13}C NMR (90.6 MHz, DMSO-d_6): δ 24.3, 103.6, 107.0, 114.1, 136.6, 145.1, 152.0, 169.1, 192.0. MS (relative intensity) (70 eV): 220.08 (11), 178.07 (M$^+$, 80), 150.08 (28), 136.06 (27), 135.06 (19), 108.07 (100), 107.06 (11), 94.01 (18), 91.04 (14). Mass calcd for $C_9H_{10}N_2O_2$ 178.0742, found 178.0747.

2,4-Diamino-1-(2-chloroacetyl)benzene (9). Chloroacetonitrile (1.30 mL, 20.5 mmol) was added to a solution of *m*-phenylenediamine (1.850 g, 17.1 mmol) dissolved in dry dichloroethane (35 mL), and the solution was cooled to 0 °C. With vigorous mechanical stirring, boron trichloride (18.8 mL, 1.0 M in heptane) was added dropwise, followed by dropwise addition of diethylaluminum chloride (10.4 mL, 1.8 M in toluene). After warming to rt, the solution was then heated and stirred for 1 h at 90 °C. A pale yellow solution with orange precipitate resulted. An equivalent volume of 2 N HCl was added, and the suspension was stirred 20 min at 90 °C. Once cooled to rt, the aqueous phase was made basic (pH 14) with 10% NaOH, and the suspension was washed with methylene chloride (5 × 100 mL). The organic fractions were combined, washed with saturated aqueous sodium chloride, and dried ($MgSO_4$), and the solvent was removed *in vacuo*. The crude material was purified by flash chromatography (47.5:47.5:5 hexane/ethyl acetate/triethyl amine) to yield 1.638 g (52%) of the product as a bronze-colored powder. Both as a solid and in solution, the product rapidly decomposes when heated. IR (KBr): 3436, 3345, 3241, 2939, 1649, 1635, 1535, 1452, 1325, 1275, 1217, 1159, 789 cm^{-1}. ^{1}H NMR (360 MHz, DMSO-d_6):

(18) At this stage, the aqueous Fluoro-NG solution may be diluted for application to microscope slides for fluorescent histochemical studies (see ref 3 above).

δ 4.67 (2H, s), 5.76 (1H, d, J = 2.2 Hz), 5.84 (1H, dd, J = 8.9, 2.2 Hz), 5.94 (2H, br s), 7.13 (2H, br s), 7.37 (1H, d, J = 8.9 Hz). ^{13}C NMR (90.6 MHz, DMSO-d_6): δ 46.5, 96.4, 104.4, 105.8, 133.2, 154.4, 154.6, 188.4. MS (relative intensity) (70 eV): 186.04 (M^+, 14), 184.04 (41), 135.05 (100), 121.08 (11). Mass calcd for $C_8H_9N_2O^{37}Cl$ 186.0374, found 186.0362.

2,4-Bis(acetylamino)-1-(2-chloroacetyl)benzene. Acetic anhydride (14.3 mL, 152 mmol) was added to a suspension of 2,4-diamino-1-(2-chloroacetyl)benzene (**9**) (1.40 g, 7.58 mmol) in methylene chloride (50 mL). After the mixture was stirred for 2 h at rt, precipitated product was noted in the green solution. The product was filtered and washed with hexane (aqua blue crystals, 1.24 g). The solvent was removed from the reaction mixture *in vacuo*, and the crude material was purified by flash chromatography (50:50 hexane/ethyl acetate) to provide a second crop of product (0.415 g, 81% total). IR (KBr): 3500–3090, 3311, 3265, 3016, 3004, 1703, 1680, 1655, 1001, 1524, 1410, 1248, 1213 cm^{-1}. ^{1}H NMR (360 MHz, DMSO-d_6): δ 2.07 (3H, s), 2.13 (3H, s), 5.09 (2H, s), 7.58 (1H, dd, J = 8.4, 1.9 Hz), 7.91 (1H, d, J = 8.4 Hz), 8.50 (1H, d, J = 1.9 Hz), 10.41 (1H, s), 11.17 (1H, s). ^{13}C NMR (90.6 MHz, DMSO-d_6): δ 24.2, 24.9, 48.2, 109.6, 112.7, 116.3, 132.3, 140.8, 145.0, 168.8, 169.3, 193.4. MS (relative intensity) (70 eV): 268.06 (M^+, 23), 220.08 (11), 219.08 (100), 177.07 (65), 135.06 (20). Mass calcd for $C_{12}H_{13}N_2O_3{}^{35}Cl$ 268.0615, found 268.0615.

1-Acetyl-6-(acetylamino)-3-indolinone (10). A solution of 2,4-bis(acetylamino)-1-(2-chloroacetyl)benzene (1.48 g, 5.50 mmol) in THF (160 mL) was added to a suspension of sodium hydride (0.211 g, 8.8 mmol) in THF (50 mL) under ice cooling. After the mixture was stirred for 4 h at rt, a 2 N HCl (50 mL) quenching agent was added with ice cooling. Aqueous sodium bicarbonate was used to return the solution pH to neutral. The mixture was washed with methylene chloride (6 × 100 mL). The organic fractions were dried ($MgSO_4$), and the solvent was removed *in vacuo* to yield 1.272 g (99%) of the product as orange powder. Mp: 220 °C dec. IR (KBr): 3252, 3191, 3117, 3077, 1680, 1603, 1561, 1437, 1387, 1260 cm^{-1}. ^{1}H NMR (360 MHz, DMSO-d_6): δ 2.08 (3H, s), 2.24 (3H, s), 4.49 (2H, s), 7.59–7.63 (2H, m), 8.63 (1H, s), 10.43 (1H, s). ^{13}C NMR (90.6 MHz, DMSO-d_6): δ 24.1, 24.3, 56.5, 106.5, 114.5, 119.7, 124.2, 147.0, 154.0, 168.6, 169.2, 193.8. MS (relative intensity) (70 eV): 232.08 (M^+, 74), 190.07 (80), 148.06 (100), 147.06 (34), 135.07 (20), 120.07 (20), 120.04 (10), 119.06 (13), 93.06 (17), 92.05 (17), 91.04 (12). Mass calcd for $C_{12}H_{12}N_2O_3$ 232.0848, found 232.0849.

3,8-Bis(acetylamino)-10*H*-quindoline (11). A solution of sodium hydroxide (0.384 g, 9.6 mmol) in 12:1 ethanol/water (25 mL) was added to 2-amino-4-(acetylamino)benzaldehyde (**7**) (286 mg, 1.60 mmol) and 1-acetyl-6-(acetylamino)-3-indolinone (**10**) (447 mg, 1.92 mmol). The dark red solution was stirred at rt for 24 h. Approximately 5 mL of 2 N HCl was added to neutralize the solution. The solvent was removed *in vacuo*, and the crude material was dissolved in DMSO (100 mL) and diluted with water (400 mL). The mixture was applied to a C_{18} reverse phase column. The column was washed with 10 and 20% methanolic water additions (50 mL each). Eluent content was monitored using a long wavelength UV lamp (in aqueous solution, the product exhibits brilliant blue fluorescence). Once the elution of the product was noted, the column was washed with methanol (800 mL). Strongly fluorescent fractions were combined and concentrated *in vacuo*. Recrystallization from 75:25 methylene chloride/methanol provided 200 mg of product as green crystals. The solvent was removed from the mother liquor *in vacuo*, and a second round of recrystallization provided an additional 61 mg of product (49% total). Mp: >260 °C. IR (KBr): 3600–2800, 1667, 1622, 1599, 1547, 1458, 1396, 1331, 1279, 1223, 1150 cm^{-1}. ^{1}H NMR (360 MHz, DMSO-d_6): δ 2.115 (3H, s), 2.125 (3H, s), 7.25 (1H, d, J = 8.5 Hz), 7.60 (1H, d, J = 8.9 Hz), 7.96 (1H, d, J = 8.9 Hz), 8.09 (1H, s), 8.12 (1H, s), 8.19 (1H, d, J = 8.5 Hz), 8.53 (1H, s), 10.19 (1H, s), 10.25 (1H, s), 11.25 (1H, s). ^{13}C NMR (90.6 MHz, DMSO-d_6): δ 30.1, 30.2, 106.8, 117.3, 118.5, 121.6, 122.1, 124.7, 127.6, 128.7, 133.5, 138.0, 143.1, 146.8, 149.9, 150.7, 151.8, 174.5, 174.6 . MS (relative intensity) (70 eV): 333.13 (21), 332.13 (M^+, 100), 291.12 (13), 290.12 (62), 289.11 (15), 249.11 (13), 248.11 (75), 247.10 (17), 220.09 (25), 161.99 (13). Mass calcd for $C_{19}H_{16}N_4O_2$ 332.1273, found 332.1259.

Fluoro-NG (1). Aqueous 1.5 N NaOH (80 mL) was added to a solution of 3,8-bis(acetylamino)-10*H*-quindoline (**11**) (360 mg, 1.08 mmol) in DMSO (40 mL). Following 3 h of stirring at 100 °C, the solution was diluted with water (200 mL) and acidified with 2 N HCl to give a dark yellow solution. Saturated aqueous sodium bicarbonate (200 mL) was then used to neutralize and buffer the resulting fluorescent yellow solution. The mixture was applied to a C_{18} reverse phase column. The column was washed with 10 and 20% methanolic water solutions. Once the elution of the product was noted by green fluorescence, the column was subsequently washed with methanol. Strongly fluorescent fractions were combined and concentrated *in vacuo* to yield 240 mg of product as orange flakes (89%). Mp: 205 °C dec. IR (KBr): 3326, 3208, 1624, 1522, 1458, 1310, 1190, 1144 cm^{-1}. ^{1}H NMR (360 MHz, DMSO-d_6): δ 5.30 (2H, br s), 5.57 (2H, br s), 6.44 (1H, dd, J = 8.4, 1.8 Hz), 6.51 (1H, d, J = 1.6 Hz), 6.86 (1H, dd, J = 8.7, 2.2 Hz), 7.01 (1H, d, J = 2.2 Hz), 7.60 (1H, d, J = 8.7 Hz), 7.70 (1H, s), 7.79 (1H, d, J = 8.4 Hz), 10.50 (1H, s). ^{1}H NMR (500 MHz, CD_3OD): δ 6.55 (1H, d, J = 1.82 Hz), 6.64 (1H, dd, J = 8.73, 1.82 Hz), 6.95 (1H, dd, J = 8.77, 1.84 Hz), 7.03 (1H, d, J = 1.84 Hz), 7.70 (1H, d, J = 8.80 Hz), 7.92 (1H, d, J = 8.73 Hz), 8.04 (1H, s). Decoupling ^{1}H NMR (500 MHz, CD_3OD): 7.92 (Irr), 6.64 (d); 7.70 (Irr), 6.95 (d); 7.03 (Irr), 6.95 (d). NOE ^{1}H NMR (500 MHz, CD_3OD): 7.92 (Irr), enhancement at 7.70. NOE ^{1}H NMR (360 MHz, DMSO-d_6): 10.50 (Irr), enhancements at 7.70, 6.51; 7.70 (Irr), enhancements at 10.50, 7.60; 6.51 (Irr), enhancement at 10.50. ^{13}C NMR (126 MHz, CD_3OD): δ 94.3, 99.7, 107.1, 112.6, 118.9, 119.4, 120.7, 124.3, 130.3, 132.1, 139.2, 139.9, 149.3, 152.2, 155.0. UV–vis (MeOH): 256 (31 300 cm^{-1} M^{-1}), 282 (21 900), 374 (13 500), 408 (13 200), 472 (15 300) nm. Excitation (MeOH): flouresence at 510 nm when excited between 250 and 500 nm. Maximum emmission with excitation at 446 nm. MS (relative intensity) (70 eV): 248.11 (M^+, 100), 247.10 (13), 221.09 (5), 220.09 (9), 124.05 (7). Mass calcd for $C_{15}H_{12}N_4$ 248.1062, found 248.1066.

Fluorescence Microscopy. Male Sprague–Dawley rats were euthanized with an overdose of chloral hydrate and promptly perfused through the aorta with 4% paraformaldehyde in 100 mM sodium phosphate buffer with pH 7.4. The brain was removed, dehydrated in 100% ethanol, and embedded overnight in poly(ethylene glycol) (MW 1400). Seven micrometer sections of the brain were slide-mounted and coverslipped with 0.000 01% Fluoro-NG in 20% glycerol, 50 mM sodium phosphate buffer, containing 0.1% *p*-phenylenediamine as an antifading agent.[19] Fluorescent staining was visualized on an epifluorescence Olympus photomicroscope using a standard fluorescein filter set and photographed. Pretreatment of sample sections with RNAse Type I (Sigma R4975), 10 mg/mL, in 50 mM Tris, 10 mM sodium chloride, and 10 mM EDTA with pH 8.0 for 30 min at 37 °C abolished staining of the neuronal perikarya and nucleolus. Control sections were stained with 0.1% cresyl echt violet, a standard Nissl stain for neuronal perikarya, and photographed with transmitted white light.

Acknowledgment. We thank the National Science Foundation (CHE-9257081), the UCLA Academic Senate, and the UCLA President's Undergraduate Fellowship for financial support of this work.

Supplementary Material Available: ^{1}H NMR and ^{13}C NMR spectra for compounds **1**, **6**, **7**, **9**, **10**, **11**, and 2,4-bis-(acetylamino)-1-(2-chloroacetyl)benzene (14 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

JO941741J

(19) Longin, A.; Souchier, C.; French, M.; Bryon, P. A. *J. Histochem. Cytochem.* **1993**, *41*, 1833–1840.