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A Long-Wavelength Biolabeling Reagent Based on the Oxonol Fluorophore

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A red fluorescent dye of the oxonol class, bis-[1-(carboxymethyl)barbituric acid-(5)]-pentamethinoxonol, has been synthesized and, in the form of the succinimidyl active ester, has been applied to antibody labeling for application to flow cytometry and to imaging of tissue sections. The new dye, named CMOX (for carboxymethyloxonol), shows maximum excitation at 583 nm and emission at 611 nm, with a quantum yield of 0.2 in aqueous buffer and methanol. Antibodies labeled with the new dye show favorable brightness, photostability, and low levels of nonspecific binding.

KEY WORDS: Oxonol succinimidyl ester; fluorescent labeling; flow cytometry; immunofluorescence.

INTRODUCTION

This laboratory has recently focused on the development of useful new fluorescent biolabels incorporating indocyanine fluorophores [1–4]. These reactive fluorescent probes provide fluorescent emission in three colors, *ca.* 580 nm (carbocyanines), *ca.* 680 nm (dicarbocyanines), and *ca* 780 nm (tricarbocyanines). For increased versatility in multicolor imaging or flow cytometry, however, it would be helpful to have additional new probes with fluorescent emission at wavelengths intermediate between those available from this family of cyanines. Previous experience [5] with a related class of dyes, the oxonols [6], suggested that a useful fluorescent probe with emission in the range of 610–620 nm might be produced by constructing a pentamethine oxonol which contained a functional group for attachment to proteins. Moreover, interest in the oxonols as a class was prompted by consideration of the fact that the oxonol fluorophore, unlike cyanines and many other red fluorophores, carries a negative rather than a positive charge and might therefore behave differently with respect to nonspecific binding.

The synthesis and testing of a succinimidyl active ester probe based on a new oxonol dye, bis-[1-carboxymethylbarbituric acid-(5)]-pentamethinoxonol (**III**) (designated CMOX, for carboxymethyloxonol), is the subject of this communication. An oxonol of a similar structure had been known previously [5], but it incorporated two *n*-octyl groups that were likely to confer an undesired tendency for nonspecific hydrophobic binding. Fluorescent labeled antibodies were easily prepared with the CMOX succinimidyl ester (**IV**), and the useful characteristics of the dyes were demonstrated when these antibodies were utilized in flow cytometry and immunofluorescent imaging studies.

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MATERIALS AND METHODS

Spectroscopic Measurements and Analytical Determinations

Spectra were determined with the following instruments: UV-VIS absorption spectra on a Hewlet Packard 8452 diode array spectrophotometer, fluorescence measurements with a Spex Fluorolog 2 or 2.5 system, IR spectra on a Nicholet Model 5DXB FT-IR spectrophotometer, and NMR spectra on an IBM NR/300 FTNMR spectrometer. Quantum yield values were determined as previously described [1]. For NMR spectra the abbreviations used are the following: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; and br, broad signal.

Synthesis of the CMOX Dye (III) and Its Succinimidyl Ester (IV)

An overview of the synthetic procedures described here is shown in Fig. 1.

Bis-[1-carboxymethylbarbituric acid-(5)]-pentamethineoxonol (III) (CMOX). 1-Carboxymethylbarbituric acid (**II**) (0.186 g, 1 mmol) (5, 8) and 0.142 g (0.5 mmol) of glutacon dialdehyde dianil (GADA) hydrochloride (**I**) [7] were dissolved in a mixture of absolute ethanol (6 ml) and triethylamine (1 ml). The mixture, which began to darken immediately, was heated at the reflux temperature for 20 min. The spectrum of a sample taken at that time indicated that nearly all of the GADA had been converted into the oxonol (maximum at 586 nm in ethanol). After the solvents had been removed in a rotary evaporator, absolute ethanol (20 ml) was added to the residue and taken off in the evaporator. The residue was then completely dissolved in water (2 ml or more) and the aqueous solution was acidified by dropwise addition with stirring of a 1 *M* aqueous solution of oxalic acid. The precipitated dye was collected by filtration and washed on the filter, first with a small amount of water, then with ethyl acetate, to produce a dark powder with a gold–green reflex. If the spectrum of the product revealed a shoulder at *ca.* 536 nm beside the 588-nm maximum, the dye was purified by reprecipitation. It was dissolved in methanol (2 ml) and the solution was diluted to 40 ml by slow addition of ethyl acetate with stirring. The precipitated product was collected by centrifugation, washed with ethyl acetate, and dried in a vacuum desiccator. Reprecipitation was repeated if the 536-nm shoulder had not been removed from the spectrum. The yield of product of satisfactory quality was 150 mg (69%). ^{1}H NMR (deuterio dimethylsulfoxide) δ=4.37 (s, 4H, $-CH_2CO_2H$), 7.51 (m, 3H, pentamethine chain), 7.70 (d, 2H, pentamethine chain), 10.63, 10.71 (2s's 1H each, acidic protons) ppm. VIS spectrum: λ_{max} 586 nm, ε 130,000 (ethanol), 582 nm (neutral water), Fluorescent emission: λ_{max} 611 nm. Formula weight for $C_{17}H_{14}N_4O_{10}$ = 434.32.

Bis-[1-carboxymethylbarbituric acid-(5)]-pentamethineoxonol Disuccinimidyl Ester (IV) (CMOX-OSu). A 0.25-mmol quantity of the oxonol dye (**III**) (0.109 g) and 0.55 mmol of *N*-hydroxysuccinimide (0.058 g) were partially dissolved in 3 ml of acetonitrile, and a solution of dicyclohexylcarbodiimide (DCC) (0.55 mmol, 0.110 g) in 2 ml of acetonitrile was added. The mixture was stirred overnight at room temperature, then centrifuged to separate the insoluble DCU by-product, which was rinsed in the centrifuge tube with additional dry acetonitrile and again removed by centrifugation. The combined acetonitrile supernatants were taken to dryness in a rotary evaporator and the dark glassy residue (purple–black with bronze reflex) was scraped from the flask with the aid of dry ether. The resulting dry powder was collected by filtration and dried. An IR spectrum was taken on a film deposited on a salt plate from methylene chloride solution. The succinimidyl ester bands at 1828 and 1799 cm^{-1} were present. The visible spectrum was the same as that of the starting oxonol. The yield was 0.144 g (91%). Formula weight for $C_{25}H_{20}N_6O_{14}$ = 628.47.

Procedures for Cell Staining with Labeled Antibodies and Flow Cytometry Measurements

Flow cytometry was performed with a dual-laser fluorescence-activated cell sorter (FACS 440, Becton Dickinson) equipped with argon and krypton lasers. Lymphocyte populations were selected based on forward and side scatter characteristics, and total T cell populations were identified using first a specified purified monoclonal antibody, CD3 MAb (Olympus, Lake Success, NY), a pan T cell marker, and then secondary goat anti-mouse IgG labeled with the red fluorophore, either CMOX or Texas red (labeled antibody obtained from Molecular Probes Inc., Eugene, OR). Labeling of the antibody by use of the succinimidyl ester **IV** was carried out by the procedure previously described for labeling of specific antibodies with succinimidyl esters of cyanine dyes [3]. The special precautions against hydrolysis recommended for use of Texas Red were not used with the CMOX reagent **IV**. Mononuclear leukocytes were obtained from the whole blood of healthy volunteers using histopaque separation medium (Sigma Chemical Co., St. Louis, MO). Indirect immunofluorescence was accomplished using the manufacturer-recommended amount of

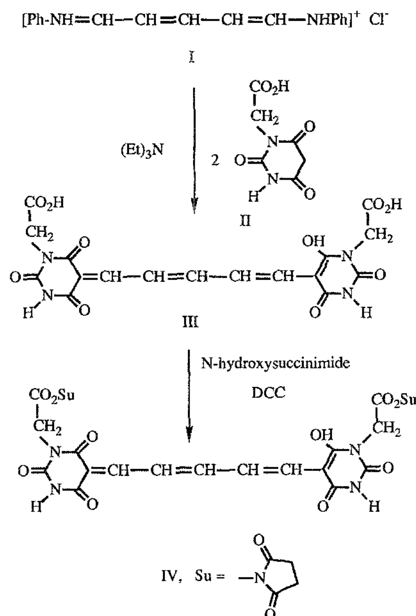

Fig. 1. Synthesis scheme for the CMOX dye (**III**) and derived active succinimidyl ester (**IV**).

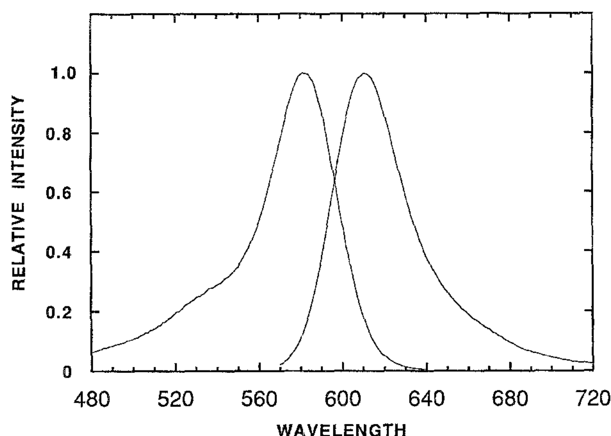

Fig. 2. Fluorescence excitation spectrum (left, emission at 650 nm) and emission spectrum (right, excitation at 560 nm) of the CMOX dye in aqueous buffer solution (pH 7.2). The spectral bandpass was 1 nm. Relative intensities are corrected. Excitation maximum is 583 nm; emission maximum, 611nm.

CD3 MAb added to 1×10^6 cells/100 µl of Hank's balanced salt solution (HBSS; GIBCO, Grand Island, NY) containing 2% fetal bovine serum and 0.1% sodium azide (monoclonal wash) and incubated at 4°C for 45 min. Optimal concentrations of secondary goat anti-mouse IgG antibodies were determined by titration. After primary and secondary antibody labeling, the cells were washed twice with monoclonal wash. Prior to analysis with the FACS 440 instrument, cell preparations

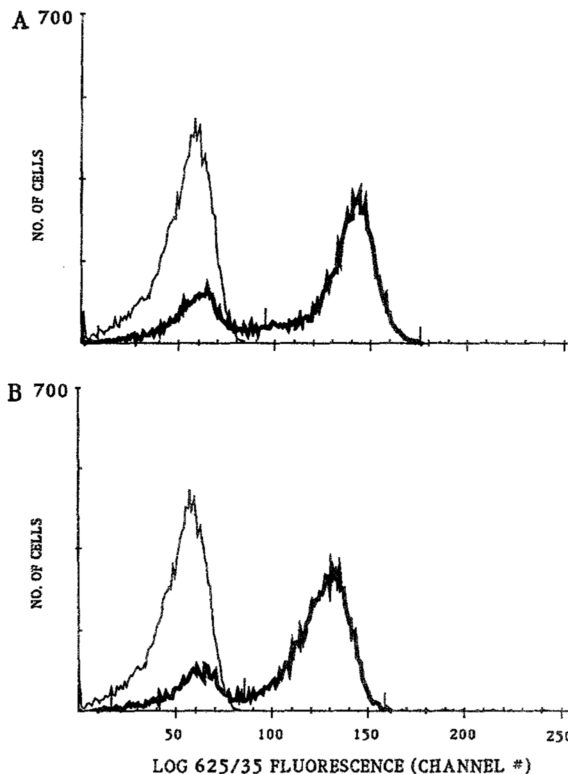

Fig. 3. Fluorescence intensity of normal peripheral blood lymphocytes examined in the flow cytometer: Cells were treated with a pan T cell monoclonal antibody (CD3), followed by treatment with fluorescently labeled goat anti-mouse IgG. (A) The result with CMOX; (B) the result with Texas Red. In each figure the heavy line plots the fluorescence of cells exposed to the labeled second antibody; the lighter line records the autofluorescence of cells treated only with the monoclonal antibody CD 3.

were fixed with 1% paraformaldehyde in HBSS. Emission from the argon laser was 400 mW at 488 nm; emission from the krypton laser was 200 mW at 568 nm. Fluorescence emission was collected through a 625/35 band pass filter. Data were recorded and analyzed using the Consort 30 VAX and DISP4 programs.

Fluorescent Labeling of Tissue Slices

Normal human cerebellum was fixed and embedded as described by Trojanowski [8]. Tissue sections were taken at 10 µm and deparaffinized. The sections were blocked in HBSS containing 0.1% sodium azide and 2% FCS (monoclonal wash) for 15 min, then incubated for 45 min at room temperature with Rmdo20 (MAb), a gift of Dr. John Trojanowski. The sections were then washed twice with monoclonal wash, followed by an incubation for 45 min with either CMOX-labeled goat anti-mouse IgG or Texas Red-labeled goat anti-mouse IgG (Molec-

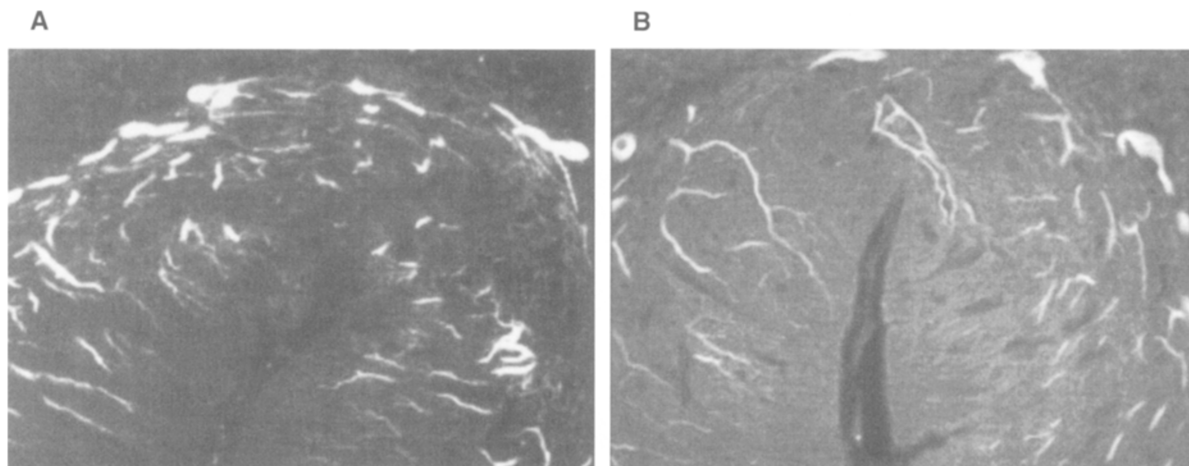

Fig. 4. Fluorescent images of human cerebellum tissue treated with a monoclonal antibody [Rmd020 (MAb)] and indirectly labeled (A) with goat anti-mouse IgG labeled with Texas Red and (B) with goat anti-mouse IgG labeled with the CMOX probe IV. Exposure times were 10 s.

ular Probes, Inc., Eugene, OR), each used at a 1:75 dilution. The sections were washed twice with HBSS, then placed in a solution of 1% paraformaldehyde solution in HBSS for 15 min. After final fixation, the sections were mounted with the aid of heat-polymerized Crystal/Mount (Biomeda Corp., Foster City, CA) and visualized via fluorescence microscopy.

Fluorescence Imaging

A multimode light microscope workstation (BDS Co., Pittsburgh, PA) equipped with a CCD (Photometrics, Ltd., Tuscon, AZ) was employed to collect and display the fluorescence emission information (see Ref. 9). The filters used in selection of excitation and emission were 558/60 and 610/40, respectively.

RESULTS

Synthesis and Fluorescence Characteristics of the CMOX Probe

Synthesis of CMOX is outlined in Fig. 1. In ethanol solutions compound **II** condensed with glutaconaldehyde dianil hydrochloride (**I**) in the presence of a base such as triethylamine to form the pentamethinoxonol **III** during a short period of heating at the reflux temperature. The oxonol **III** gives bluish–red solutions (absorption maximum at 586 mn in ethanol, ε, 130,000) with fluorescent emission in the red at 611 nm. The progress of dye formation was monitored spectroscopically; it was necessary to limit the duration of the heating period to minimize the formation of a second dye of undetermined identity which shows its absorption maximum at 490 nm. The reactive fluorescent probe **IV**, the disuccinimidyl ester of **III**, was obtained in good yield by treatment of **III** with *N*-hydroxysuccinimide and dicyclohexylcarbodiimide.

Emission and excitation spectra of the CMOX dye are shown in Fig. 2. The fluorescence quantum yield of the free dye in aqueous solution or methanol was 0.2. It declined to as low as 0.09 when several CMOX fluorophores were linked to a protein.

Flow Cytometry with the CMOX Probe

In flow cytometry trials, lymphocyte T cell populations were identically treated with a primary mouse antibody and then with either of two goat anti-mouse IgG preparations, one labeled with CMOX in this laboratory and the other labeled with Texas Red by Molecular Probes, Inc. (Eugene, Oregon), and supplied commercially. The results of the flow cytometry comparison are shown in Fig. 3. The mean channel number for red fluorescent emission was 138.1 from CMOX-labeled cells, compared to 125.6 for Texas Red-labeled cells. This is a comparison on a logarithmic scale which corresponds to a measured brightness for the CMOX-labeled cells of 1.68 times that for the Texas Red-labeled cells.

Application of CMOX to Immunofluorescence Microscopy

The results of an immunofluorescence imaging application of the CMOX-labeled goat anti-mouse anti-

body are shown in Fig. 4. Shown are images obtained by fluorescence light microscopy from mouse cerebellum tissue under 10-s excitation. CMOX-labeled antibody provided bright and high-contrast images of the fluorescently stained Purkinje cells and a low level of nonspecific background fluorescence. The CMOX images compared favorably with those taken using Texas Red-labeled antibody.

The utility of fluorescent dyes in microscopy is limited by their rate of photolysis during observation of the fluorescent image. CMOX showed a photostability comparable to that of Texas Red in the above immunofluorescence experiments. Photostability can be significantly affected by the slide mounting medium used. Although CMOX and Texas Red were used without difficulty with Crystal/Mount, they were markedly more stable in the UV-cured Norland Optical Adhesive 81 (Norland Products, Inc., New Brunswick, NJ).

DISCUSSION

In work on biological specimens the excitation and observation of long wavelength fluorescence can be achieved with a minimum of interference from the autofluorescence that would be produced by excitation at shorter wavelengths. Red fluorophores can therefore have a particular value in the construction of biological probes. In this paper, we explore the applicability of the oxonol fluorophore for this purpose. An oxonol with the requisite red fluorescent emission and labeling properties was prepared utilizing 1-carboxymethylbarbituric acid (**II**), a recently described compound which was created to serve as the source of new calcium indicators of the purpurate (murexide) type [11,12]. The dye synthesis was accomplished in one step from intermediates which are not difficult to prepare, and the reactive disuccinimidyl ester derivative was easily obtained by treatment with *N*-hydroxysuccinimide and dicyclohexylcarbdiimide (DCC).

The new reagent, CMOX, showed useful properties when tested in biological applications involving labeling and application of antibodies. Labeling of proteins was carried out in alkaline buffer solutions by the same simple procedures customarily used with other succinimidyl esters, and no special precautions were necessary to avert hydrolysis of the reagent. In flow cytometry studies, CMOX antibodies provided bright and specific staining, comparable to that obtained from antibody labelled with Texas Red, a commonly used reagent with similar fluorescence wavelengths. In immunohistochemistry applications, CMOX-labeled antibodies also produced images with brightness and contrast comparable to those produced using Texas Red antibodies. These experiments indicate that the CMOX probe holds promise in applications requiring long-wavelength fluorescence labeling.

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