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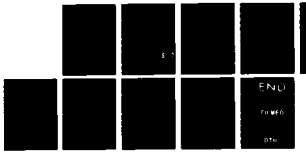

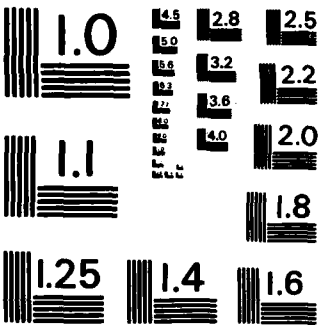

AD-A162 725

BIMANE DERIVATIVES AS FLUORESCENT PROBES FOR BIOLOGICAL MACROMOLECULES

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1.Scientific Results

The primary aims of our research on the bimanes are divided into the following areas: a) synthesis b) reactivity c) photophysics d) application to macromolecules. Although we have a number of new results, it seems pertinent to base this report on a new summary of the application of bimane labeling to thiols. The next report will cover chemistry and photophysics.

The title of the report (a chapter) is: Thiol Labeling with Bromobimanes by Nechama S. Kosower and Edward M. Kosower. The chapter is attached.

2. Research Plans

We are continuing work on the fluorescence lifetimes of bimanes. The research on the fluorination of a bimane will appear in a current J. Org. Chem. We still desire to prepare syn-(H,F)B. Although we have been blocked by a shortage of starting material, new ideas for its synthesis have encouraged us to start again in January 1986. Successful research on vinyl and tricyclic bimanes will be described in the next report.

3. Meetings and other news.

Prof.E.M.Kosower lectured at Brookhaven National Laboratory, the Experimental Station of DuPont and at the University of California, San Diego during the summer of 1985. The bimanes bibliography now contains 61 references and will be updated in the next report.

Thiol Labeling with Bromobimanes

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Thiol Labeling with Bromobimanes

Nechama S. Kosower and Edward M. Kosower

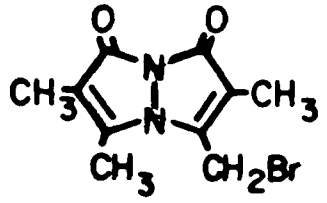
Some nine years ago, we discovered and developed convenient syntheses for a new class of heterocyclic molecules, the bimanes or 1,5-diazabicyclo[3.3.0.] octadienediones.[1-3] In the course of preparing the bromo derivatives, we found fortuitously that proteins were fluorescently labeled by bromobimanes. We soon established that bromobimanes reacted preferentially with thiols, and had demonstrated the usefulness of such labeling for both small and large molecules in biological systems.[4-9] Many applications of the agents have been reported.[13-24] Of special importance is the development by Fahey et al of HPLC methods for the quantitative determination of individual thiols at the picomole level in non-protein fractions of biological systems, methods which have seen considerable use.(see Fahey et al, this vol.)[10-12,21-24]

Fluorescent labeling agents[25] ideally add minimal size fluorescent moieties ("labels") to target molecules, usually proteins, but sometimes nucleic acids or polysaccharides. The smaller the label, the less likely it is to change the biological, physical and chemical properties of the labeled molecule as compared with the unlabeled molecule. This principle is apart from the changes expected because the groups (such as thiols) which are labeled are themselves central to a particular activity. Fluorescent labels are preferred over radioactive labels (in cases for which either may be used) because of the simplicity of detection and the avoidance of the necessity for safe disposal of radioactive materials.

Bromobimanes are derived from a basic structure (two fused five-membered rings) with the requisite minimal size. This chapter describes the use of

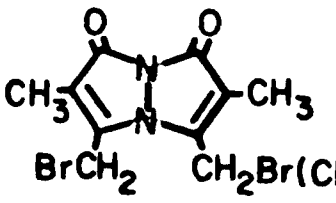
three bromobimanes for fluorescent labeling of biological systems. The three bromobimanes,[26] mBBr (1), bBBr (2) and qBBr (3), are commercially available[27]. The short form[3] names are given under the formulas.

Chemical and Photophysical Background



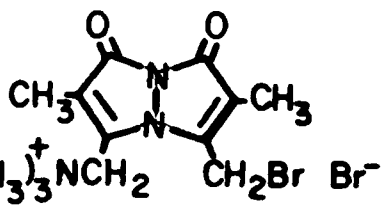
1

mBBr
syn-($BrCH_2$,CH_3)(CH_3,CH_3)B



2

bBBr
syn-($BrCH_2$,CH_3)B



3

qBBr
syn-($BrCH_2$,CH_3)((CH_3)$_3N^+CH_2$,CH_3)B,Br^-

Two isomeric series of bimanes are known, those with the carbonyl oxygens on the same side (syn-) and those with the carbonyl oxygens on opposite sides (anti-). The syn-series is generally fluorescent, the anti-series phosphorescent. The use of the latter in biological systems remains to be studied.

The neutral agents are moderately soluble in medium polarity organic solvents (acetonitrile, dichloromethane), and slightly soluble in water. The quaternary salt, qBBr, is soluble in water, but less soluble in organic solvents. Bromobimanes are yellow. The absorption maxima for the agents and those of some derivatives are given along with emission maxima in Table 1.

The bromoderivatives are essentially non-fluorescent, and are relatively stable when stored in the dark. Chromatography on thin-layer silica yields a yellow nonfluorescent spot which develops a blue fluorescence after several minutes exposure to 360nm light. The change is a convenient characteristic for identification and also suggests that there is some sensitivity to light, with the nature of the photchemical processes still undetermined.

The reactions of bromobimanes with the tripeptide thiol, glutathione

(GSH) are second order and dependent on pH, the active nucleophile being the thiolate anion, GS^-.[28] The reaction of bromobimane with a thiolate (eqs.1,2) converts the nonfluorescent agent into a water-soluble fluorescent derivative, which also has an absorption spectrum different from that of the agent.

(1) mBBr + RS^- ----> mBSR + Br^-

(2) bBBr + $2RS^-$ ----> RSBSR

Bromobimanes are less reactive towards other nucleophiles (amines, carboxylates), especially in neutral aqueous solution in which the amines are largely in the ammonium ion form. Nevertheless, two points must be considered. (1) Buffer anions are much less active as nucleophiles than thiolate anions, but are present in much greater concentrations. Therefore, nonnucleophilic buffers should be used and dilute buffers are to be preferred. (2) In a labeling reaction involving multifunctional molecules like proteins, the first step involves displacement of the bromine of bBBr by a thiol, but the second bromine may react with a less reactive nucleophilic group if its local concentration is high, an expression of the neighboring group effect.

Biological properties of bromobimanes and derivatives

The bromobimanes, mBBr (1) and bBBr (2), are neutral, and easily penetrate intact, live cells; positively charged qBBr (3) does not penetrate the live cell. Alkaline conditions are required for many other fluorescent labeling agents, such as those with isothiocyanate or sulfonyl chloride groups for linking. In contrast, bromobimanes can be used at physiological pH, exhibiting fast reactions with small thiols[28] (complete labeling of intracellular nonprotein thiols (NPSH) in sec to min) and somewhat slower reactions with protein thiols (min to hours). One may thus not only identify reactive

thiol species but also learn how accessible the reactive groups are under physiological conditions.

Monobromobimane, mBBr, is the most frequently used in the series, not only as a labeling agent for thiols, but also for studies on the consequences of the alkylation of reactive thiols in biological systems. In analyses, mBBr may be added to intact cells before processing, obviating oxidative loss of thiols during fraction preparation. In addition to total thiol determination, the mBBr procedure makes possible quantitation of thiols in individual proteins and NPSH (see the chapter by Fahey et al, this volume[12b]).

The bisbromobimane, bBBr, is used for cross-linking of reactive groups within the same chain or between different polypeptides in isolated proteins or in the intact cell (see below).[6,7,28-30]

The charged bromobimane, qBBr, labels extracellular thiols as well as whole dead cells or lysed cells; the labeled material can be taken up by some live cells via endocytosis or phagocytosis.

The bimane-labeled materials are stable to air, to light, to chemical and biochemical procedures, and are resistant to irradiation (e.g., do not fade, or fade very slowly during observation with fluorescence microscope). Labeled materials exhibit good quantum yields of fluorescence and the small fluorescent moiety (being only two pentacyclic rings), minimally perturbs macromolecular conformation.

Labeling Procedures

Bromobimane solutions: Stock solutions of mBBr and bBBr in acetonitrile (a chemically and biochemically inert) are easily prepared with concentrations of 50-150 mM. The acetonitrile solutions are stable at room temperature,

provided they are kept in the dark (exposure to light causes some photolysis, with some conversion to a fluorescent bimane). Small volumes of the stock solutions are used in labeling experiments. mBBr can be diluted in aqueous media *prior to use (see below)*, but bBBr, which is less soluble in aqueous media, is usually not diluted. qBBr is dissolved directly in buffer immediately before use. Some buffers may contain reactive nucleophiles (see remarks under chemistry) and should be checked for the extent of reaction with bromobimanes before use.

Reactions in solution

A solution of small thiol or protein in buffer is added with rapid mixing to a small volume of bromobimane stock solution. The mBBr can be first diluted with buffer before mixing. The reaction vessel should be protected from light to avoid photolysis. The reaction can be followed by changes in light absorption or fluorescence emission using aliquots which are clear, colorless solutions. The reaction can be stopped by the addition of acid. In the case of mBBr and bBBr, the reaction can also be terminated by extraction of the unreacted reagents from the aqueous reaction mixture with CH_2Cl_2. The fluorescence of the products from certain proteins (hemoglobin, chlorophyll-associated proteins) is measured after removal of the chromophore. Aliquots of the reaction mixture are mixed with 0.15N HCl/acetone or 2.5% oxalic acid/acetone, the colorless, precipitated protein washed and then redissolved for fluorescence measurements.

To expose thiol groups which are conformationally inaccessible in macromolecules dissolved in the usual aqueous buffers, solvents such as DMSO may be added to the media used for the reaction with mBBr.[16]

Reactions in cells and tissues

A cell suspension in buffer is added with rapid mixing to an appropriate bromobimane solution, the amounts and reaction times varying according to the cell type and number and purpose of labeling. As an example, most of the bromobimane-reactive thiols in human red cells are labeled by adding one ml of a 10% cell suspension to 15-25 µl of 100 mM mBBr in acetonitrile and incubating the suspension for 30-45 min at 37°C.[6,8,32] For labeling of live spermatozoa, a suspension of 5-10 $x10^6$ cell/ml is incubated with 0.05-0.1 mM mBBr for 5-10 min, conditions which do not interfere with sperm motility or fertilizing ability.[17,33] Final concentrations of 0.05-0.5 mM mBBr are used for labeling of reactive thiols in various other cell suspensions, cell monolayers and subcellular fractions, e.g. bacteria, lymphocyte suspensions, fibroblasts, chloroplast suspensions.

Reaction can be terminated by washing of the samples, by the addition of acid or by extracting residual free mBBr or bBBr with CH_2Cl_2.

The labeling of protein thiols in tissues is conveniently carried out by treating tissue sections with mBBr.[9] Suitable samples include sections of frozen tissues dried and fixed on slides and paraffin sections of fixed tissues, after treatment with xylene to remove the paraffin and washing with ethanol. Sections on slides are washed with buffer, covered with 0.1 mM mBBr solution in buffer and left in the dark at room temperature for 5-30 min. Sections are then washed and covered with glycerol and coverslips for microscopic observations. Once the labeling reaction is over, the slides can be kept in the light without any precautions. A similar procedure is used for fixed subcellular fractions on slides, e.g., chromosome preparations.[34]

In addition to thiol labeling in tissues, mBBr can be used for the

histochemical detection of disulfides. Tissue sections are first covered with 10-50 mM NEM solution and incubated for a few min in order to block free SH groups, washed with buffer and treated with 10-50 mM DTT for 5-10 min. Slides are then washed thoroughly with buffer and labeled with mBBr as above. To label both thiols and disulfides, slides are treated only with DTT and washed prior to mBBr treatment.[9]

Analytical procedures

Spectroscopic data for bromobimanes and bimane-labeled products

Spectroscopic data (absorption maxima, absorption intensities, fluorescence maxima and quantum yields) of bromobimanes and bimane-labeled products are given in Table 1. The values vary somewhat from material to material and should be determined for accurate analyses of particular derivatives.

Fluorescence microscopy.

Cells, cell fractions and tissue sections can be examined with an epiillumination type fluorescence microscope, equipped with appropriate filters for excitation between 360-400 nm, and emission at wavelengths longer than 440-450 nm. Photographs are made with sensitive films such as Kodak Tri-X or Ektachrome. In most cases, thorough observations and photography are possible, without an appreciable decrease in fluorescence intensity.

Absorption and fluorescence measurements of cells and cell fractions

The total reactive thiol content can be measured in whole samples or in any cellular fraction, provided clear, colorless solutions are obtained (e.g., SDS-dissolved whole cells, chlorophyll-free chloroplast stromal proteins and Coupling Factor (CF_1), non-protein fractions, etc). For quantitative determination, the fluorescence intensity is compared with that of a known labeled

small thiol or protein such as labeled globin.

Individual thiol-containing protein bands are readily observed by fluorescence following seperation by gel electrophoresis. Gels (fixed by standard methods) are viewed using a long wavelength ultraviolet lamp ("black light"). Gels are photographed by placing them on a long wavelength ultraviolet transilluminating box, using a Kodak Wratten gelatin filter no. 8 (fixed gels may be stored in the fixative for days before photography). After fluorescence photography, the gels are stained with dyes such as Coomassie blue . A densitometric profile of fluorescent and stained protein bands may be obtained, using either the gels or films of the gels. The thiol content of the proteins in the bands is quantitated by comparison with the profile from a labeled known protein run on the same gel.

The thiol content of protein fractions separated by column chromatography can be measured by the mBBr procedure. Thiol-containing peptides can be identified in peptide maps following enzymatic degradation of labeled protein.

Other Applications of Thiol Labeling

A. Non-thiol molecules can be converted to thiol-containing derivatives, then labeled with mBBr and used as probes and fluorescent substrates. Examples are:

a) $(Fab)_2$ was reduced by dithiothreitol (DTT), then reacted with mBBr.[35] The labeled fragment has been used in immunocytochemical experiments.[35]

b) Pepstatin was converted to an SH derivative (by coupling to cysteamine), treated with mBBr and the mB-derivative used as a fluorescent probe for the subcellular location of cathepsin D with selectivity for the active conformation of the enzyme.[36]

c) $tRNA_f^{Met}$, containing thiouridine, was reacted with mBBr and the mB-derivative shown to be useful for studying the tRNA-ribosome interaction.[16]

B. The dynamics of microtubule assembly and disassembly can be studied. Labeling of assembled microtubules was carried out with qBBr.[37] The derivatized proteins appeared to undergo association-dissociation in the same way as the unlabeled materials.[37]

C. The bimane moiety may serve as a component of a donor-acceptor system in energy transfer studies, as in the case of rhodopsin.[13]

D. Substrate for glutathione transferase. mBBr was shown to react at least 3 times faster with GSH in the presence of GSH transferase.[38]

E. Immobilization of motile cells. mB-labeling of spermatozoa had no effect on sperm motility. Excitation of the labeled sperm by fluorescent microscopy resulted in almost instantanous immobilization of the spermatozoa. Immobilization depended on the midpiece being irradiated, sugesting a mitochondrial site of action.[33]

F. Diversion of electrons generated in chloroplast photosystem II (PSII). The mBBr is converted to a radical anion, $mBBr^{\cdot -}$, by electron transfer from the plastoquinone anion of PSII. Although most of the radical anion, $mBBr^{\cdot -}$, is converted to <u>syn</u>-(CH_3,CH_3)B via protonation and further reduction, a significant proportion yields a reactive free radical via dissociation of bromide ion. The free radical reacts with a PSII protein to yield a sB-protein. [39]

G. For formation of a sulfide from two thiols via reaction with syn-(1-bromoethyl,methyl)bimane. Glutathione sulfide (GSG) has been produced in this way from glutathione.[40]

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25 The class description for such agents is somewhat confusing. In general, fluorescent labeling agents are compounds which give rise to fluorescent derivatives of target molecules. The latter thus carry fluorescent labels. The agents themselves may or may not be fluorescent.

26 Formal names are: mBBr, 4-bromomethyl-3,6,7-trimethyl-1,5-diazabicyc-

lo[3.3.0]octa-3,6-dien-2,8-dione; bBBr, 4,6-bis(bromomethyl)-3,7-dimethyl-1,5-diazabicyclo[3.3.0]octa-3,6-dien-2,8-dione; qBBr, 4-bromomethyl-3,7-dimethyl-6-trimethylammoniomethyl-1,5-diazabicyclo[3.3.0]octa-3,6-dien-2,8-dione

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40 A.E.Radkowsky, E.M.Kosower, D.Eisenberg and I.Goldberg, submitted for publication

Table 1. Absorbance and Fluorescence of bromobimanes and labeled materials

Bromobimanes and sB-labeled[a] materials	Absorbance $\lambda_m(\epsilon_m)$		Fluorescence
	CH_3CN	buffer[b]	$\lambda_m(\phi_F)$[c]
mBBr	381 (6000)	396 (5300)[d]	(0)[e]
bBBr	392 (6000)	--	(0)
qBBr	--	378 (5700)[f]	(0)
mB-globin[g]	--	385 (~4200)	484 (~0.18-0.23)
mB-SR(MPA)[h]	--	392	484 (~0.26-0.28)
mB-SG[i]	--	390 (~5300)	482 (~0.3)
bB-globin[g]	--	385 (~4000)	477 (~0.25-0.33)
qB-globin[g]	--	370 (~4000)	480 (~0.08-0.13)
qB-SR(MPA)[h]	--	378	475 (~0.08)
qB-SG[i]	--	375 (~5300)	482 (~0.07-0.09)

[a]sB-labeled is a general term for any of the syn-bimane products. mBBr, bBBr and qBBr yield the mB-, bB-, and qB-labeled materials, respectively.

[b]Buffer containing 135 mM NaCl-10 mM Phosphate, pH 7.4.

[c]Quantum yield of fluorescence was based on quinine sulfate fluorescence as reference (ϕ_F).

[d]An mBBr solution in acetonitrile was diluted with buffer[b].

[e]The quantum yields of fluorescence of the bromobimanes are < 0.001. However, measurement must be done with proper care since photolysis may give rise to some fluorescence.

[f]Dissolved in buffer immediately before measurements.

[g]Labeled globin was prepared from hemoglobin reacted with bromobimane. It was stored as an air-dried powder and dissolved in H_2O for measurements.

[h]Metaphosphoric acid extract (MPA) of labeled erythrocytes, which contain NPSH, mainly GSH (see chapter by Fahey et al, this volume (ref.12b)).

[i]Labeled GSH prepared by reacting GSH in buffer.

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