



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁴ : A61K 43/00, 49/00, 49/02 C07D 311/82	A1	(11) International Publication Number: WO 87/ 06138 (43) International Publication Date: 22 October 1987 (22.10.87)
(21) International Application Number: PCT/US87/00819 (22) International Filing Date: 7 April 1987 (07.04.87) (31) Priority Application Number: 850,845 (32) Priority Date: 11 April 1986 (11.04.86) (33) Priority Country: US (71) Applicant: PRESIDENT AND FELLOWS OF HARVARD COLLEGE [US/US]; 17 Quincy Street, Cambridge, MA 02138 (US). (72) Inventors: KINSEY, Berma, McDowell ; 44 Lombard Street, Newton, MA 02158 (US). KASSIS, Amin, I. ; 57 Stockdale Road, Needham, MA 02192 (US). AD-ELSTEIN, Stanley, James ; 63 Gammons Road, Waban, MA 02168 (US).		(74) Agent: FURLONG, Robert, W.; One Financial Center, Boston, MA 02111 (US). (81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent). Published <i>With international search report.</i>
(54) Title: DIHYDRORHODAMINES AND HALOGENATED DERIVATIVES THEREOF (57) Abstract Dihydrorhodamines can be made by reduction of rhodamines and can be covalently halogenated by conventional procedures. The compounds are useful as labelling, imaging, and therapeutic agents for mammalian cells.		

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AT	Austria	FR	France	ML	Mali
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DIHYDRORHODAMINES AND HALOGENATED DERIVATIVES THEREOF

This invention relates to novel dihydrorhodamines and to covalently halogenated labelling, imaging compounds or therapeutic compounds and pertains more specifically to radiohalogenated dihydrorhodamine compounds. The compounds of the invention can be used for detecting, labelling, imaging, or therapeutically treating viable mammalian cells. They are of particular value for use with certain types of tumor cells because of their increased retention by such cells as compared to retention by normal cells.

It has previously been proposed to use rhodamine 123, a permeant cationic dye as a mitochondrial stain of viable mammalian cells and as a probe for the study of mitochondrial function and membrane potential. This compound is retained longer in certain types of tumor cells than in normal cells and exhibits selective toxicity to certain tumor cells in vitro and in vivo.

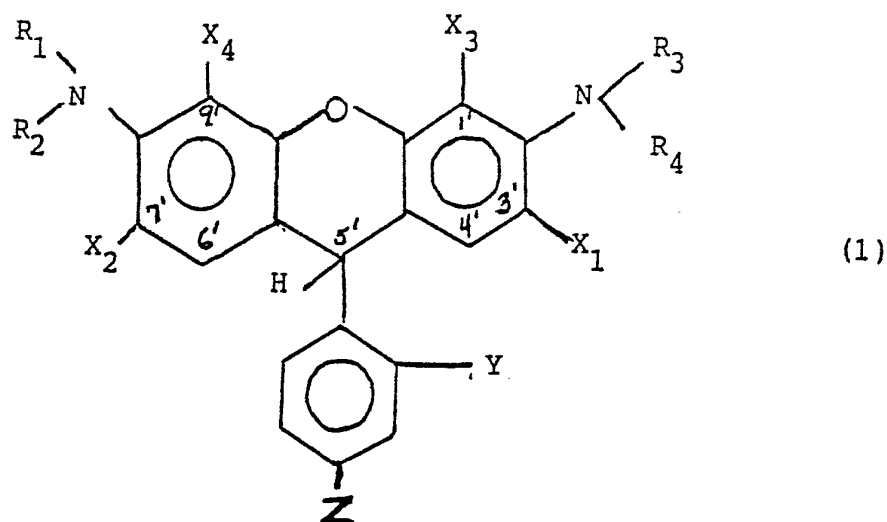
It has previously been proposed by Padmanabhan et al., J.Nucl.Med., Vol. 26, 124 (1985) and by Thakur et al., 5th Int.Symp. on Radiopharm.Chem., 11-p-7 (1984) that rhodamine 123 be radioiodinated with ^{125}I and ^{131}I and used as an imaging or radiotherapeutic agent. However, it has now been found that the positive charge on the quaternary nitrogen atom of the rhodamine in solution prevents the formation of covalently iodinated rhodamines; instead, conventional iodination procedures such as the chloramine T or iodogen procedures lead to the formation of the corresponding quaternary ammonium iodide, an ionic compound in which the iodine is subject to ionization and replacement by other non-radioactive ions in an aqueous medium such as a biological fluid.

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It has also been reported by Gallop et al.,
Biotechniques, Vol. 2, pp.32-36 (1984) that hydrogenated
ethidium bromide is taken up by and reoxidized inside
viable mammalian cells.

5 It has now been found that dihydrorhodamines
and halogenated dihydrorhodamines are taken up, oxidized
to the fluorescent parent rhodamines, and retained by
mammalian cells. Furthermore, unlike the rhodamines
themselves, the dihydrorhodamines can be covalently
10 halogenated by conventional halogenation procedures, and
the halogen of such compounds is internalized by the
cells, thus providing the cells with enhanced
susceptibility to toxic radiation. Consequently,
halogenated and radiohalogenated dihydrorhodamines are
15 useful as detecting, labelling, imaging or therapeutic
agents.

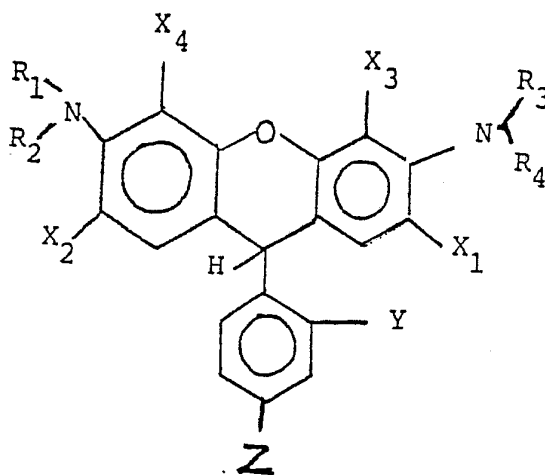
The present invention comprises a compound
having the structure



20 in which R_1, R_2, R_3, R_4 and R_5 are hydrogen or
lower alkyl (preferably having 1-5 carbon atoms), and

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X_1, X_2, X_3 and X_4 are hydrogen, lower alkyl (preferably having 1-5 carbon atoms), halogen, or a radiohalogen, Y is $-\text{COOR}_5$ or $-\text{SO}_3\text{R}_5$, Z is hydrogen, $-\text{COOR}_5$, or $-\text{SO}_3\text{R}_5$, and water-soluble salts of such compounds. Preferably the salts are those which are physiologically acceptable; they include, for example, the chloride perchlorate, tetrafluoroborate, and internal salts where, for example, both Y and Z are $-\text{SO}_3\text{H}$. The invention also comprises bringing into contact with a mammalian cell an aqueous solution comprising a compound having the structure

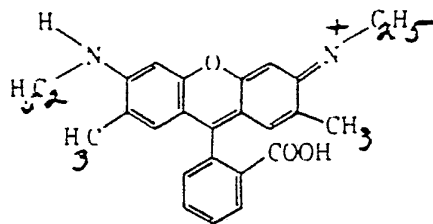


in which R_1-R_5 , X_1-X_4 , and Y and Z are as defined above, to cause said compound to be taken up by the cell. Preferably at least one of X_1 , X_2 , X_3 and X_4 is halogen or radiohalogen, the remainder being hydrogen.

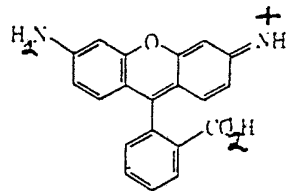
The dihydorhodamine compounds of the present invention can be made by hydrogenation or reduction of the corresponding rhodamine compound by conventional procedures such as by reduction with a borohydride, e.g. sodium borohydride in aqueous medium. Among such

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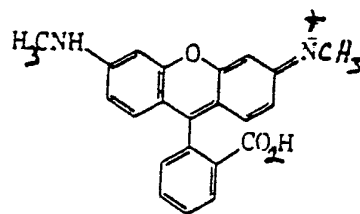
corresponding rhodamine compounds are rhodamine B, rhodamine 3B, rhodamine 6-G, rhodamine 19, rhodamine 110, rhodamine 116, rhodamine 123, sulforhodamine B, and including various water-soluble salts. The structure of rhodamine 19 is



while that of rhodamine 110 is



and that of rhodamine 116 is



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The dihydrorhodamine products have the structure (1) given above in which X_1, X_2, X_3 and X_4 are hydrogen. The dihydrorhodamines can be covalently halogenated by any conventional procedure such as the chloramine T, iodogen, or N-chlorosuccinimide procedure, or any other procedure effective to introduce one or more atoms of chlorine, bromine, iodine or astatine into an aromatic amine. The products, containing halogen covalently bonded in any one or more of the four available positions X_1-X_4 in the amino substituted benzene rings can, if desired, be separated from each other by chromatography. However, separation is not essential and mixtures containing two or more different halogenated compounds are useful in the same way as the individually pure compounds.

The dihydrorhodamine compounds after halogenation with a halogen radioisotope such as chlorine, bromine, iodine or astatine may be used as labelling, imaging or therapeutic agents in the same way as rhodamine 123 or any other such agent simply by dissolving the compound in an aqueous medium such as normal saline or any physiologically acceptable medium and bringing it into contact with the viable mammalian cells it is desired to treat, whereupon the compounds are taken up by the cells and located in the cytoplasm.

The following specific examples are intended to illustrate more fully the nature of the present invention without acting as a limitation upon its scope.

Example 1

Dihydrorhodamine 123: Rhodamine 123 hydrochloride (100 mg, 0.26 mmol; Eastman Kodak) was dissolved in 50 ml of water and 50 ml of CH_2Cl_2 was added. Excess solid $NaBH_4$ was added in portions with

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vigorous magnetic stirring until almost all the color was discharged. After 1 hr, the pale orange organic layer was separated and the water layer extracted twice with CH_2Cl_2 . The combined organic layers were
5 passed through anhydrous Na_2SO_4 and evaporated under reduced pressure. The crude oily reaction mixture was purified by flash chromatography (C) using 10% ethylacetate in CH_2Cl_2 as eluent, giving 80 mg of a slightly pinkish-orange oil (88% yield). The purified
10 oil was dissolved in a small amount of CH_2Cl_2 and precipitated with hexane. The pinkish-white solid dihydrorhodamine 123 was washed with hexane, and a portion submitted for analysis. (m.p. = 163-165°C; calc. for $\text{C}_{21}\text{H}_{18}\text{N}_2\text{O}_3$: 72.96% C, 5.50% H, 7.88% N; found: 72.82% C, 5.24% H, 8.09% N). ^1H NMR (CDCl_3) σ = 3.93 (s, $-\text{CO}_2\text{CH}_3$), 5.93 (s, C5'), 6.15 (d of d, J = 2, 8, 2H, C7', 3'), 6.30 (d, 2H, C9', 1'), 6.70 (d, 2H, C6', 4'), 7.0-7.17 (m, 3H, C3, 4, 5), 7.65 (d of d, 1H, C6). The product possessed the structure
20 (1) above in which $\text{R}_1, \text{R}_2, \text{R}_3$, and R_4 are hydrogen, R_5 is methyl, and $\text{X}_1, \text{X}_2, \text{X}_3$ and X_4 are hydrogen.

Example 2

Iododihydrorhodamine 123 (A): Dihydrorhodamine
25 123 (43 mg, 0.12 mmol) was dissolved in 50 mL of CCl_4 and 1 mL of CH_2Cl_2 , and cooled in ice under red light. A solution of 17 mg NaI in 0.5 mL MeOH and 0.5 mL 0.2 M NH_4OAc was added with stirring and a cold solution of 17 mg (0.13 mmol) of N-chlorosuccinimide in
30 50 mL CCl_4 was added in small portions. The reaction mixture was allowed to warm to room temperature and stirring was continued for 1 hr. Water and NaBH_4 were added, the organic layer separated, dried over anhydrous

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Na_2SO_4 and evaporated under reduced pressure. The red oily product was subjected to flash chromatography using 5% EtOAc in CH_2Cl_2 , and 26 mg of A (44%) along with 7 mg of C (9%) were recovered. A sample was dissolved in a minimal amount of ether, precipitated with hexane, and submitted for analysis. (calc. for $\text{C}_{21}\text{H}_{17}\text{IN}_2\text{O}_3$: 53.41% C, 3.63% H, 26.87% I; found: 53.21% C, 3.97% H, 27.08% I). ^1H NMR (CDCl_3): σ = 3.88 (s, $-\text{CO}_2\text{CH}_3$), 5.93 (s, C-5'), 6.15 (d of d, $J=3$, 8 Hz, C-7'), 6.28 (d, $J=3$, C-9'), 6.38 (s, C-1'), 6.67 (d, $J=8$ Hz, C-6'), 7.15 (s, C-4'), 6.97-7.20 (m, C-3,4,5), 7.67 (m, C-6).

3'-Iododihydrorhodamine 123 (A), 1'-iododihydrorhodamine 123 (B), 3',7'-diiododihydrorhodamine 123 (C), 1',7'-diiododihydrorhodamine 123 (D): A solution of 86.0 mg (0.25 mmol) of dihydrorhodamine in 100 mL CCl_4 was prepared with the addition of 3-4 mL of CH_2Cl_2 . A solution of 150 mg (1.0 mmol) NaI in 5 mL of MeOH and 5 mL of 0.2 M NH_4OAc was added and the mixture cooled in an ice bath with stirring. A cold solution of 133.5 mg (1.0 mmol) of N-chlorosuccinimide in 100 mL of CCl_4 was added all at once and the mixture stirred in an ice bath for 0.5 hr. Stirring was continued at room temperature for 0.5 hr., after which water and a small amount of solid NaBH_4 were added. The organic layer was separated after vigorous shaking, and the water layer washed several times with CH_2Cl_2 . The combined organic layers were filtered through anhydrous Na_2SO_4 and the solvent removed under reduced pressure. Flash chromatography on a 1.5 cm column was done on the residue, using 85 mL of 1% EtOH in CH_2Cl_2 , 50 mL of 5% EtOH in CH_2Cl_2 , and 40 mL of 10% EtOH in CH_2Cl_2 , collecting 5 mL

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fractions. Four products were isolated and characterized by NMR.

A: 23 mg, 21% yield, R_f (in 5% EtOH/ CH_2Cl_2) = 0.35. Identical with previously prepared A.

5 B: 13 mg, 11% yield, R_f (in 5% EtOH/ CH_2Cl_2) = 0.60. (calc. for $\text{C}_{20}\text{H}_{15}\text{I}_2\text{N}_2\text{O}_3$: 53.41% C, 3.63% H, 26.87% I; found: 53.31% C, 3.92% H, 26.08% I). $^1\text{HNMR}$ (CDCl_3): $\sigma = 3.98$ (s, $-\text{CO}_2\text{CH}_3$), 6.08 (s, C-5), 6.30 (d of d, $J = 3, 8$ Hz, C-7'), 6.35 (d, $J = 8$ Hz, C-3'), 6.56 (d, $J = 3$ Hz, C-9'), 6.78 (m, C-4'6'), 6.88-7.42 (m, C-3,4,5), 7.22 (m, C-6).

10 C: 26 mg, 17% yield, R_f (in 5% EtOH/ CH_2Cl_2) = 0.72. (calc. for $\text{C}_{20}\text{H}_{14}\text{I}_2\text{N}_2\text{O}_3$: 42.17% C, 2.70% H, 42.43% I; found 42.98% C, 3.04% H, 41.70% I). $^1\text{HNMR}$ (CDCl_3): $\sigma = 4.00$ (s, $-\text{CO}_2\text{CH}_3$), 6.05 (s, C-5'), 6.43 (s, C-1',9'), 7.22 (s, C-4',6'), 6.98-7.23 (m, C-3,4,5), 7.75 (d of d, $J = 2, 7$ Hz, C-6).

20 D: 18 mg, 12% yield, R_f (in 5% EtOH/ CH_2Cl_2) = 0.87. (calc. for $\text{C}_{20}\text{H}_{14}\text{I}_2\text{N}_2\text{O}_3$: 42.17% C, 2.70% H, 42.43% I; found: 41.20% C, 2.90% H, 42.72% I). $^1\text{HNMR}$ (CDCl_3): $\sigma = 3.97$ (s, $-\text{CO}_2\text{CH}_3$), 6.08 (s, C-5'), 6.32 (d, $J = 8$ Hz, C-3'), 6.62 (s, C-9'), 6.73 (d, $J = 8$ Hz, C-4'), 7.22 (s, C-6'), 6.87-7.33 (m, C-3,4,5), 7.73 (d of d, $J = 3, 8$ Hz, C-6).

25 1',3',7'-Triiododihydrorhodamine (E): To a solution of 86 mg (0.25 mmol) of dihydrorhodamine in 20 mL of methanol and 6 mL of 0.2 M NH_4OH was added a solution of 187.5 mg (1.25 mmol) NaI in 10 mL of methanol and 2.5 mL of 0.2 M NH_4OH . Nitrogen was bubbled into the stirred solution and 150 mg (0.35 mmol,

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1.4 meq) of Iodogen was added all at once. Stirring was continued under N_2 for 1 hr. The reaction mixture was filtered, and methanol removed from the filtrate under reduced pressure. The filter cake was washed several times with CH_2Cl_2 , and the water layer extracted several times with CH_2Cl_2 . The CH_2Cl_2 fractions were combined and the solvent removed under reduced pressure. The residue was dissolved in 2-3 mL of CH_2Cl_2 , absorbed onto a small amount of silica gel, and the CH_2Cl_2 removed under reduced pressure. The dry silica gel reaction mixture was applied to the top of a 1.5 cm column for flash chromatography using 25% EtOH in hexane. Three main products were recovered: 26 mg (17%) of C (R_f in 25% EtOH in hexane = 0.27; 11 mg (7%) of D (R_f = 0.43), and 14 mg (8%) of E (R_f = 0.58). (calc. for $C_{20}H_{13}I_3N_2O_3$: 34.84% C, 2.09% H, 52.58% I; found: 34.61% C, 2.30% H, 53.73% I). 1H NMR ($CDCl_3$): σ = 3.98 (s, $-CO_2CH_3$), 6.10 (s, C-5'), 6.58 (s, C-1'), 7.22 (s, C-4',6'), 6.80-7.30 (m, C-3,4,5), 7.75 (m, C-6).

Example 3

A 2.5 mM solution of the dihydrorhodamine of Example 1 in CCl_4 with the addition of a few drops of CH_2Cl_2 was prepared, and to one mL of this solution was added the contents of two vials of $Na^{125}I$ (14.2 mCi). The vials were rinsed twice with 10 μ L of 0.2 M NH_4OAc and 10 μ L of methanol, and the resulting mixture stoppered and stirred in an ice bath. A 2.5 mM solution of N-chlorosuccinimide in CCl_4 was added and the vial stoppered, covered with aluminum foil, and stirred vigorously at room temperature for 1 hr. One mL of water and several 10 μ L portions of 1M $NaBH_4$ were added and stirring continued until most of the red color

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had disappeared. The CCl_4 layer was removed with a pipet and the remaining water layer was washed twice with small amounts of CH_2Cl_2 . Solvent was evaporated from the combined organic layers under a stream of nitrogen, and one mL of methanol and a tiny amount of solid NaBH_4 were added to colored residue. The methanol was evaporated, and the now colorless residue was taken up in 5% EtOAc/ CH_2Cl_2 and flash chromatography performed using the same solvent on a 0.8 x 12 cm column, 85 drops per fraction. Fractions which showed a single spot on TLC radioautography were combined to give 4.22 mCi of A (Table 1), 1.36 mCi of B, 1.49 mCi of C, and 0.40 mCi of D. Fractions showing more than one spot on TLC were combined and rechromatographed using 25% EtOAc/hexane. From this chromatography was isolated 0.18 mCi of B, 0.53 mCi of C, 0.16 mCi of D, 0.09 mCi of F, and 0.02 mCi of unidentified material with a greater R_f on TLC than F. The total radioiodinated yield was 8.45 mCi (60%) of which 50% was A, 18% was B, 24% was C, 7% was D, and 1% was E.

TABLE 1

Compound	X1	X2	X3	X4	R1	R2	R3	R4	R5
A	I	H	H	H	H	H	H	H	CH ₃
B	H	H	I	H	H	H	H	H	CH ₃
C	I	I	H	H	H	H	H	H	CH ₃
D	I	H	H	I	H	H	H	H	CH ₃
E	I	I	I	H	H	H	H	H	CH ₃

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In order to characterize the individual compounds present in each fraction, the same reaction had been carried out using non-radioactive sodium iodide and individual fractions obtained by chromatography were characterized by NMR. The fractions containing ^{125}I were thus identified by comparison on TLC with NMR-authenticated material.

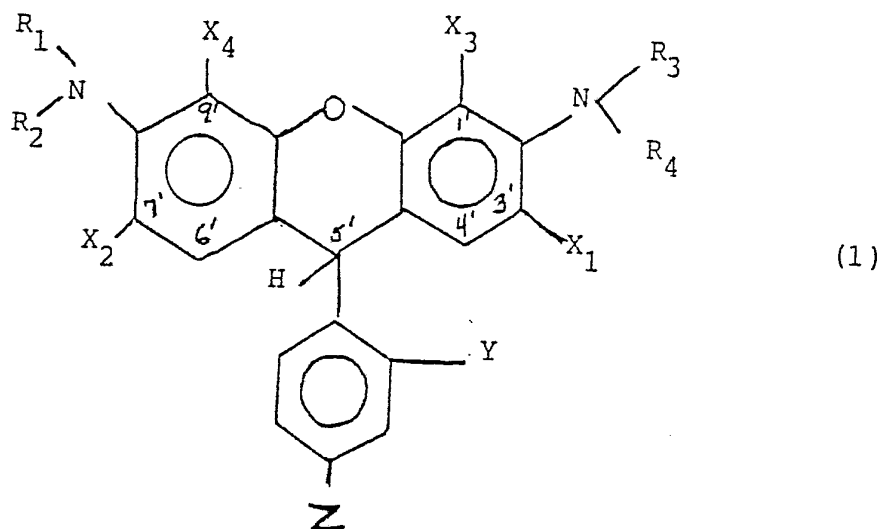
Other dihydrorhodamines, and halogenated and radiohalogenated derivatives thereof can be made by similar procedures.

While dihydrorhodamine 123 is itself a nonfluorescent compound, when an aqueous solution of the compound is applied to viable mammalian cells, its presence in the mitochondria of the cells is observed by fluorescent microscopy, showing that it is oxidized within the cell to the fluorescent rhodamine 123. Halogenation of the compound does not interfere with its being taken up by the cells and oxidized.

What is claimed is:

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1. A compound having the structure



in which R_1, R_2, R_3, R_4 and R_5 are hydrogen or lower alkyl, at least one of X_1, X_2, X_3 and X_4 is halogen or radiohalogen, the remainder being hydrogen or lower alkyl, Y is $-COOR_5$ or $-SO_3R_5$ and Z is hydrogen, $-COOR_5$, or $-SO_3R_5$, and water-soluble salts of such compounds.

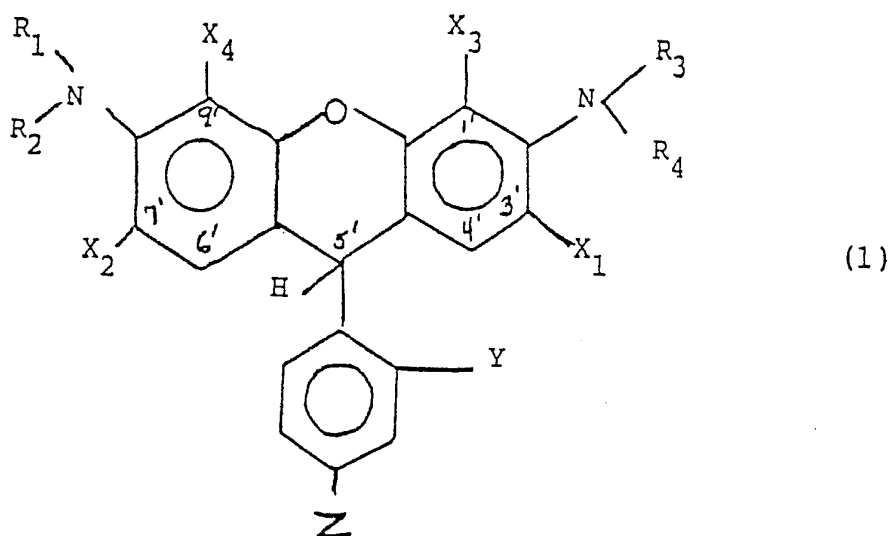
2. A compound as claimed in claim 1 in which at least one of X_1, X_2, X_3 and X_4 is a radioisotope of chlorine, bromine, iodine or astatine, the remainder being hydrogen.

3. A compound as claimed in claim 1 in which R_1, R_2, R_3 and R_4 are hydrogen and R_5 is methyl, Y is $-COOR_5$, and Z is hydrogen.

4. A compound as claimed in claim 3 in which at least one of X_1, X_2, X_3 and X_4 is a halogen radioisotope of chlorine, bromine, iodine or astatine.

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5. The method which comprises bringing into contact with a mammalian cell an aqueous solution comprising a compound having the structure



5 in which R_1, R_2, R_3, R_4 and R_5 are hydrogen or lower alkyl, at least one of X_1, X_2, X_3 and X_4 is halogen or radiohalogen, the remainder being hydrogen or lower alkyl, Y is $-\text{COOR}_5$ or $-\text{SO}_3\text{R}_5$ and Z is hydrogen, $-\text{COOR}_5$, or $-\text{SO}_3\text{R}_5$, and water-soluble salts of such compounds.

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6. The method as claimed in claim 5 in which R_1, R_2, R_3 and R_4 are hydrogen, R_5 is methyl, Z is hydrogen and at least one of X_1, X_2, X_3 and X_4 is a radioisotope of chlorine, bromine, iodine or astatine, the remainder being hydrogen.

5

7. The method as claimed in claim 6 in which at least one of X_1, X_2, X_3 and X_4 is a radioisotope of iodine.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US87/00819

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ³		
According to International Patent Classification (IPC) or to both National Classification and IPC IPC(4): A61K 43/00, 49/00, 49/02; C07D 311/82 U.S. Cl.: 424/1.1, 3, 7.1; 435/29, 34; 549/227, 394		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁴		
Classification System	Classification Symbols	
U.S.	549/227, 394, 424/1.1, 3, 7.1; 435/29, 34	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁶		
STN INTERNATIONAL STRUCTURE SEARCH		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴		
Category *	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
Y	Journal of Labelled Compounds and Radio-pharmaceuticals, Volume 21, Nos. 11-12, issued 1984 (England), International Symposium on Radiopharmaceutical Chemistry, Tokyo, 9-13 July, 1984 Symposium Abstracts, M.L. Thakur, "Mitochondria Specific Rhodamine 123: Radioiodination and Preliminary Evaluation as an Agent for Scintigraphy and Radiotherapy of Certain Tumors", see pages 1120-1122.	1-7
Y	The Journal of Nuclear Medicine, Volume 26, issued 1985 (New York), Proceedings of the 32nd Annual Meeting, Poster session No. 529, S. Padmanabhan, "Rhodamine-123: Radioiodination and Evaluation as an Agent for Imaging and Radiotherapy of Certain Tumors", see page P124.	1-7
Y	Biotechniques, Volume 2, No. 1, issued January/February 1984, (Natick, Massachusetts), Paul M. Gallop, "Dynamic Approaches to the Delivery of Reporter Reagents into Living Cells", see pages 32-36.	1-7
* Special categories of cited documents: ¹⁵ "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "&" document member of the same patent family		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search ² 29 JUNE, 1987		Date of Mailing of this International Search Report ² 08 JUL 1987
International Searching Authority ¹ ISA/US		Signature of Authorized Officer ²⁰ <i>Norma S. Milestone</i> Norma S. Milestone

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

- | | | |
|---|--|-----|
| Y | E.N. Abrahart "Dyes and their Intermediates", published 1968, by Pergamon Press (London), see pages 223-225. | 1-7 |
| Y | Colour Index, third edition, Volume 4, published 1971 by The Society of Dyers and Colourists (Great Britain), see pages 4417, 4431-4432. | 1-7 |

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹⁰

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers , because they relate to subject matter ¹² not required to be searched by this Authority, namely:

2. ☐ Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out ¹³, specifically:

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ¹¹

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No ¹⁸
Y	U.S., A, 4,306,014 (KUNIKANE), 15 December 1981.	1,3
Y	U.S., A, 4,067,840 (WOLF), 01 January 1978.	1-7
A	U.S., A, 4,298,591 (O'BRIEN), 03 November 1981.	
A	U.S., A, 3,743,713 (KATO), 03 July, 1973.	
A	U.S., A, 3,728,351 (COUNSELL), 17 April, 1973.	
A	U.S., A, 4,256,727 (TRIPLETT), 17 March, 1981.	
A	DT, C, 44,002 (BADISCHE ANILINE- UND SODA-FABRIK), 06 June 1888.	
Y	DT, C, 49,057 (FARBWERKE), 14 September, 1889.	1-7