FORM 25 (12) PATENT granted by (19)

A P165

AFRICAN REGIONAL INDUSTRIAL PROPERTY ORGANIZATION (ARIPO)

(11) A

(21) Application Number: AP/P/90/00195

(22) Filing Date: 26.07.90

(24) (45) Date of Grant& Publication 12.01.92

(30) Priority Data:

(33) Country: US

(31) Number: 07/386 904

(32) Date: 28.07.89

(84) Designated States:

BW GH KE MW
SD UG ZW

(73) Applicant(s):

BECTON, DICKINSON AND COMPANY
ONE BECTON DRIVE
FRANKLIN LAKES
NEW JERSEY 07417-1880
U.S.A.

(72) Inventor(s):

LINDA LEE
1397 LLOYD WAY
MOUNTAIN VIEW
CALIFORNIA
U.S.A.
(SEE OVERLEAF)

(74) Representative:

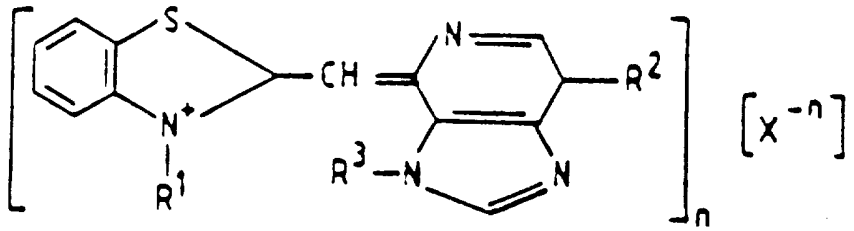
HONEY & BLANCKENBERG
P O BOX 85
HARARE
ZIMBABWE

(51) International Patent Classification Int. Cl.[5] C09B 23/04

(54) Title: FLUORESCENT NUCLEUS ACID DYES

(57) Abstract:

Dyes, capable of preferentially staining nucleic acids, have the formula:



in which R^1, R^2 and R^3 are the same or are different and each is $-CH_3$ or $-CH_2COO^-$; X^- is an anion and n is an integer.

A P165

(56) Documents cited: GB 2 190 189 A
DE-OS 3 839 397 A1
GB 2 114 542 A
US 4 544 546

INVENTOR

PATRICK MIZE
2113 SUMMIT STREET
DURHAM
NORTH CAROLINA
U.S.A.

M&C FOLIO: 799P61084 WANGDOC: 1089i

NOVEL FLUORESCENT DYE

This invention relates to a novel fluorescent dye, and, more particularly, relates to a novel fluoresecent dye which preferentially stains nucleic acids, the use of such dye and the preparation thereof.

Blood-borne parasite infections present a major health problem in many areas of the world. Many of these areas lack both the equipment and skilled technicians to operate the equipment that are available for the detection of such parasites in a biological sample such as blood or a component thereof. In order to combat these problems, certain low-cost and low-skill level instruments have been developed but which provide accurate easily readable results. One such instrument comprises a capillary tube which contains a generally cylindrical mass having a specific gravity such that it will float in one of the cell layers when a blood sample is separated by centrifugation. The mass is selected such that it will form a thin annular space in the tube into which the parasite bearing cells will be crowded, thus increasing he concentration of parasites in a restricted area. The tube then is examined with the aid of a microscope for the presence of parasites within the

annular region. US patent No 4,190,328 describes one such device employing this method. Commercially, the QBC system (Becton Dickinson Primary Care Diagnostics) embodies this method.

A drawback to this method, however, is that absent the addition of a stain to highlight the presence of the parasite in any given cell the detection of such parasites may be difficult. Parasites may go through several different developmental stages in a particular host. Discriminating between stages is often difficult and requires a certain degree of skill and practice. The presence or absence of a particular stage may be indicative of the relative severity or stage of the infection.

In US Patent No 4, 190,328, acridine orange is disclosed as a membrane permeable stain that will stain the nucleic acids of parasites. Acridine orange, however, also is permeable in other blood cells and thus will stain to some degree nucleated white blood cells. Thus, where the clinician is not skilled in the identification of the stages of an infection, false positives may occur using a stain like acridine orange.

Another method for the analysis of blood-borne parasites is not applicable to field use but is

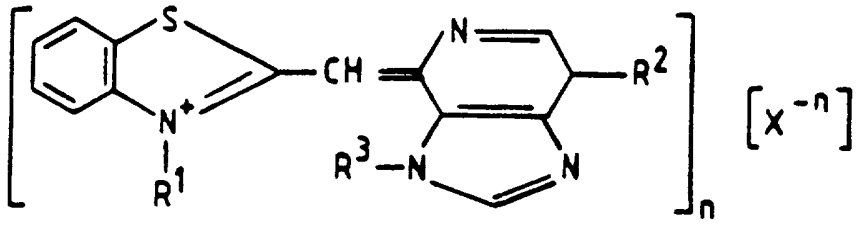
applicable to research use. This method comprises the use of a flow cytometer and a membrane permeable, nucleic acid stain such as thiazole orange. This method recently was described by Makler et al, Cytometry, 8:568 (1987).

Generally, this method comprises isolating a whole blood sample from a patient and staining the cells with thiazole orange. The stained cells then are run through the flow cytometer such as a FACScan (Becton Dickinson Immunocytometry Systems). As the cells pass through the flow cytometer, they pass through a sensing region, substantially one at a time, wherein each cell is scanned by a light of excitation wavelength, typically light at 488nm from an argon laser. Light scattered by and fluorescent light emitted from each cell are detected by sensing means, such as photodetectors, and each cell is identified based upon all the light signals detected.

As noted in the reference, background staining of nucleated cells (both immature reds and all stages of whites) will occur as will staining of platelets. Although the staining of white blood cells can be "gated" out of the cell analysis, staining of the nucleated red cells and platelets cannot be gated out and thus will provide background fluorescence which may effect the identification or parasite bearing cells.

Accordingly, what is required for the improved practice of a method such as those described above is a stain that preferentially stains the nucleic acids of blood-borne parasites with little or no staining of nucleated red and white blood cells and platelets. It has now been found, in accordance with the present invention that certain dyes, as hereinafter defined, are capable of such preferential staining.

Accordingly, one embodiment of the invention provides nucleic acid dyes of the general formula:



in which R^1, R^2 and R^3 are the same or are different and each is a $-CH_3$ or $-CH_2COO^-$ group; X^- is an anion (such as a halide, an inorganic ion such as PO_3^{3-}, SO_4^{2-}, IO_4^-, $C10_4^-$, NO_3^-, NO_2^- or the like, and an organic ion such as acetate, glucose-6-phosphate, D-glucuronate and the like); and n is an integer (the valency of the anion X).

The dye is excitable at 488nm (with maximal excitation at 460nm) and emits fluorescence in the presence of nucleic acids between 470 and 550nm with a maximum emission at 478nm. The dye selectively stains both RNA and DNA nucleic acids.

In the following description, reference will be made to the accompanying drawings in which: -

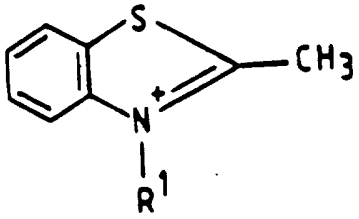
Figure 1 is a plot of absorbance versus wavelength (nm) for a solution of the iodide salt of a dye of the invention with and without RNA; and

Figure 2 is a plot of fluorescence versus wavelength for a solution of the p-toluenesulfonate salt of a dye of the invention with and without RNA.

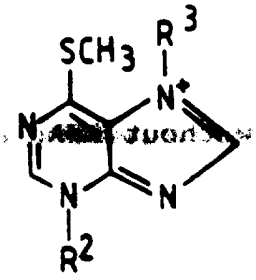
The present invention comprises a novel flurorescent dye that preferentially will stain the nucleic acids. It is excitable at 488nm and emits maximally at 478nm. In the presence of RNA, the fluorescence enhancement of the dye is greater 7,000 fold. The dye has a quantum yield of approximately 0.4.

The invention also provides a method of staining nucleic acids in a biological sample (especially blood or a component thereof) which method comprises contacting the sample with a dye in accordance with the invention.

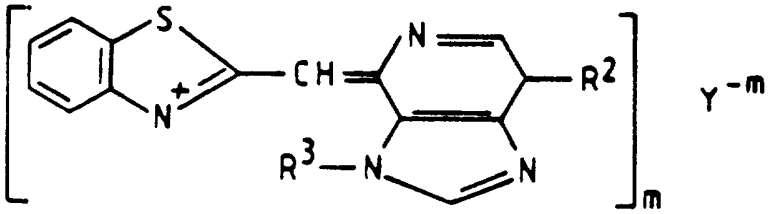
The invention further provides a process for the preparation of a dye of the invention which process comprises reacting a compound of the formula:



(in which R^1 has the meaning defined above) in the form of a salt with an anion, with a compound of the formula:



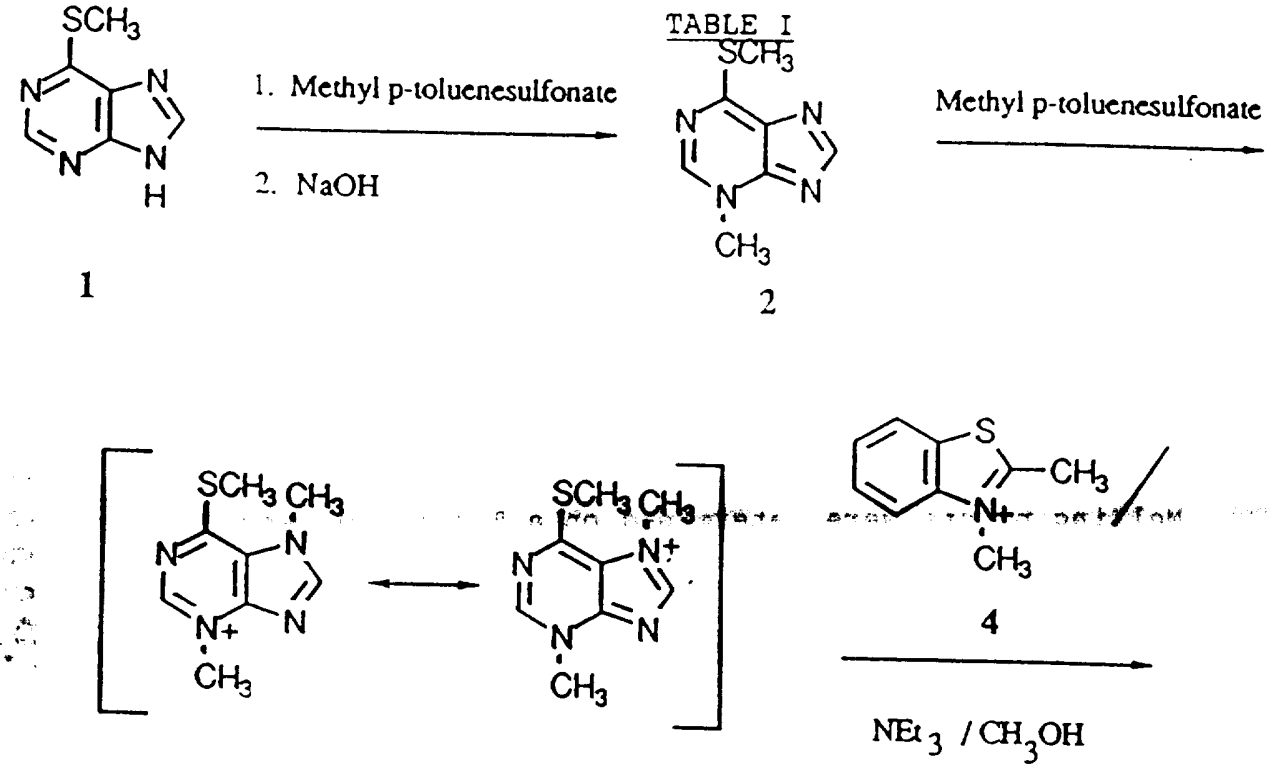
(in which R^2 and R^3 have the meanings defined above) also in the form of a salt with an anion, in the presence of an acid binding agent, to yield a compound of the formula:

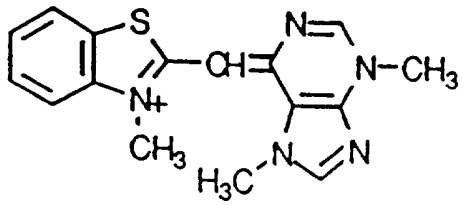


(in which R^1, R^2 and R^3 have the meanings as defined above, Y is an anion and m is an integer); and subsequently; if desired, converting the anion Y to another anion, X.

In order that the invention may be well understood the following examples are given by way of illustration only. The dye was synthesized in the following manner which is further set forth in Table I. Unless otherwise specified, all compounds mentioned herein are obtainable from Aldrich Chemical Co. Intermediates 2 and 3 were prepared by minor modification of the methods set forth in Neiman et al, Israel J Chem 3: 161 (1965). Intermediate 4 was prepared by the method of Brooker et al, J. Am. Chem. Soc., 67: 1889 (1945).

Melting points were determined on a Thomas Hoover capillary melting point apparatus and are uncorrected. NMR spectra were recorded on an IBM WP-200SY and chemical shifts were reported relative to tetramethylsilane. Analytical reverse phase ion paired HPLC was performed on a Waters 860 two pump system with photo diode array detection (200-600nm) using a Brownlee cyano 4.6 x 220mm column with the following conditions: nitial hold for 5 minutes at 50mM triethylammonium acetate in water at pH 6.5 followed by a linear gradient to 50mM triethylammonium acetate n acetonitrile over a 1 hour period. High resolution mass spectra were obtained from Mass Spectrometry Facility of Duke University.


SCH3
1. Methyl p-toluenesulfonate
2. NaOH
TABLE I
SCH3
Methyl p-toluenesulfonate
CH3
1
2
SCH3 CH3
CH3
3
S
CH3
CH3
4
NEt3 / CH3OH


S
CH
N
N-CH3
CH3
H3C
N

Pur-1

a) Preparation of 3-Methyl-6-(methylthio)purine (2):

A round bottomed flask was charged with 3gm of 6-(methylthio)purine (1), 3.7gm of methyl-p-toluenesulfonate and 6ml of dimethylforamide. The mixture was heated in an oil bath at 110°C for 2.5 hours until it became a clear yellowish solution.

After cooling, 20ml of water was added to the solution which then was extracted with three 20ml portions of ether to remove unreacted starting material. The combined ethereal portions were back-extracted with 20ml of water, the aqueous layer was washed with 20ml ether and then combined with the initial aqueous solution. The solution then was made basic to pH 13 by the addition of KOH. After several minutes, a white crystalline solid precipitated from solution. The solid was filtered and washed with water and air dried. The white crystals were identified as 3-methyl-6-(methylthio)purine (2).

b. Preparation of 3,7-Dimethyl-6-(methylthio)purine p-toluenesulfonate (3):

A round bottomed flask was charged with 0.80gm of 3-methyl-6-(methylthio)purine (2) and with 0.95gm of methyl p-toluenesulfonate. The mixture was briefly heated in an oil bath at 100°C. The homogeneous

solution was cooled and then washed with acetone and ether. After washing, the reaction mixture appeared as an amorphous white solid. The organic washes were combined with a white crystalline solid formed. This material (3) was combined with the amorphous solid and was used without purification in the synthesis of PUR-1.

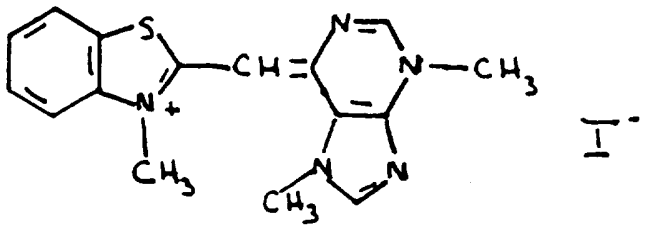
(c) Preparatioon of 2,3-Dimethylbenzothiazolium iodide (4):

4.8 gm of methyl iodide and 5 gm of 2-methylbenzothiazole were combined in a round bottomed flask equipped with a metal stirring bar and reflux condenser. The flask was heated to 80°C in an oil bath for 16 hours. The pinkish-white solid (4) was cooled, crushed, washed with acetone and filtered.

(d) Preparation of PUR-1

A round bottomed flask, equipped with a magnetic stirring bar and a reflux condenser, was charged with 1.28 gm of 2,3-dimethylbenzothiazolium iodide (4), approximately 1.50 gm (4.4mM) of crude 3,7-dimethyl-6-(methylthio)purinium-p-toluenesulfonate (3), 20 ml of methanol and 0.5 ml of triethylamine. The mixture was refluxed for about 45 minutes, producing a red solution containing a yellow solid. The material was cooled,

filtered and washed with methanol and ether resulting in a yellow-orange solid that was identified by the formula:



This material was identified as a 3-methyl-2-[(3,7-dimethyl-6-purinylidene)-methyl]-benzothiazolium (PUR-1). PUR-1 is the preferred embodiment of the stain and has the following characteristics: mp= 340-345°C; nmr (CD_3OD) δ 3.97 (s, 3H), 3.99 (s, 3H), 4.27 (s, 3H), 6.56 (s, 1H), 7.39 (t, 1H), 7.57 (d, 1H), 7.80 (d, 1H), 8.04 (d, 1H), 8.54 (s, 1H) and 8.85 (s, 1H); HPLC (30 minute retention time for one component), UV max. = 448nm; high resolution FAB-MS for $C_{16}H_{16}N_{05}S_{01}I_{01}$ (M^+) calculated - 310.1126, found - 310.1136.

The p-toluenesulfonate salt was prepared by substituting 2,3-dimethylbenzothiazolium p-toluenesulfonate for 2,3-dimethylbenzothiazolium iodide. The p-toluenesulfonate salt of PUR-1 had the following characteristics when identified by 200 MHz NMR spectroscopy; (CD_3OD) δ 8.66 (s, 1H); 8.31 (s, 1H); 7.9-7.2 (m, 8H); 6.55 (s, 1H); 4.28 (s, 3H); 4.03 (s, 3H); 3.98 (s, 3H); 2.30 (s, 3H).

Other salt forms may be made from the iodide form by an anion exchange procedure. Briefly, eluent was pumped through a Brownlee Aquapore anion 10 x 250mm column at 2.0 ml/min. The column was flushed with approximately 100ml of water and was followed by an equilibration with 10 ml of a 1.0M solution of the sodium salt of the anion to be exchanged. Excess buffer was flushed from the column with 200ml of water. The iodide salt of the stain was injected onto the column and eluted with water of 1:1 solution of water and acetonitrile.

Using this method the following salts of PUR-1 were made as set form in Table II:

TABLE II

Equilibrating salt	Eluting solvent	Final Salt Form
1.0M $NaPHO_4$	water	(PUR-1)$NaPHO_4$
1.0M $NaSO_4$	water	(PUR-1)$NaSaO_4$
1.0M Na oxalate	water; acetonitrile	(PUR-1)-D-oxalate
1.0m Na-D-glucuronate	water: acetonitrile	(PUR-1)-D-glucuronate

It was found that the $PO_4 3^-$ and SO_4^{2-} forms of PUR-1 formed by this method were more soluble

than the iodide form of PUR-1. Accordingly, it may be more desirable to use these forms of PUR-1 than the iodide form when coating capillary tubes into which blood or other blood components later will be added for analysis.

The solubility of the iodide form of PUR-1 also may be improved by substituting CH_2COO^- for any or all of the methyl groups attached to nitrogen. This may be accomplished by substituting bromo acetic acid for methyl p-toluenesulfonate in the synthesis of compound 3.

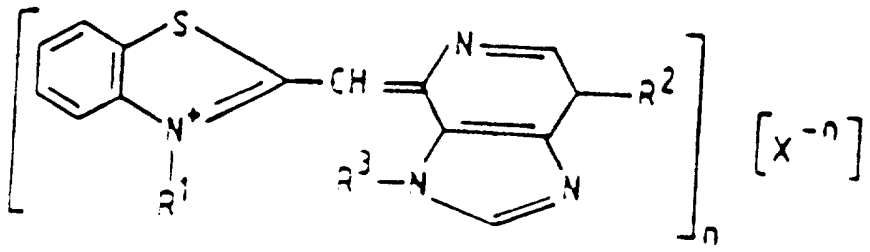
Referring to FIG. 1, a 1mM solution of p-toluenesulfonate salt of PUR-1 in methanol was prepared. The solution was diluted to a concentration of 2×10^{-5} M in phosphate buffered saline ("PBS") or to a concentration of 2×10^{-5} M in a solution of PBS containing RNA (torula yeast, Sigma Chemical Co.) at a concentration of 1mg/ml. The absorbance maximum in the absnece of RNA was 448nm ($\varepsilon = 6.3 \times 10^4 M^{-1} cm^{-1}$), and the absorbance maximum in the presence of RNA was 459nm ($\varepsilon = 6.0 \times 10^4 m^{-1} cm^{-1}$).

Referring to FIG. 2, a solution of PUR-1 in PBS (100μM) was prepared. Toa 3ml cuvette was added 2.97 ml PBS and 30ml of the PUR-1 solution. The fluorescence emission fo the solution was measured with an excitation

wavelength of 460nm. No fluorescence was observbed int he absence of RNA. To a second cuvette was added 2.97 ml of PBS, RNA solution (1 mg/ml, 0.30 ml) and 30 µl of the PUR-1 solution. Fluorescence emission was measured as above. A broad emission curve was measured with a maximum at 478nm and an approximate quantum yield of 0.4.

Claims: -

1. Compounds of the formula:



in which R^1, R^2 and R^3 are the same or are different and each is $-CH_3$ or $-CH_2COOH$; X^{-n} is an anion and n is an integer.

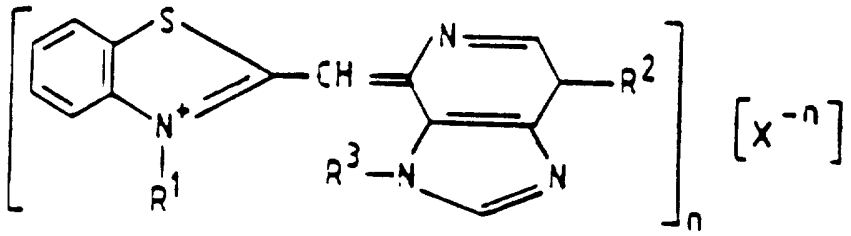
2. Compounds as claimed in claim 1 wherein the anion is a halide.

3. Compounds as claimed in claim 1 wherein the anion is PO_4^{3-}, SO_4^{2-}, IO_4^- ClO_4^-, NO_3^- or NO_2^-.

4. Compounds as claimed in claim 1 wherein the anion is acetate, glucose-6-phosphate D-glucuronate.

5. A compound as claimed in claim 2 wherein X^- is I^- and R^1, R^2 and R^3 are each $-CH_3$.

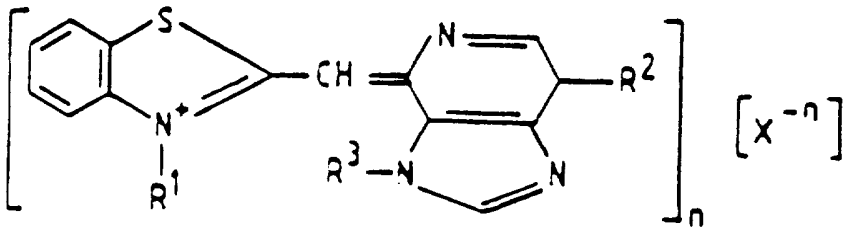
7. A method of staining nucleic acids in a biological sample comprising contacting said sample with a compound of the formula:



in which R^1, R^2 and R^3 are the same or are different and each is $-CH_3$ or $-CH_2COO^-$; X^- is an anion and n is an integer.

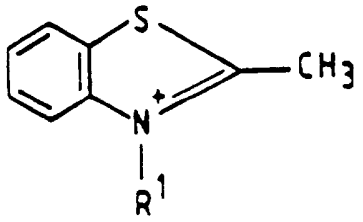
8. A method as claimed in claim 7 wherein the sample is blood or a component thereof.

9. A process for the preparation of a compound of the formula:

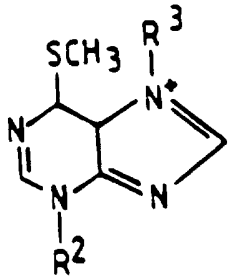


(in which R^1, R^2 and R^3 are the same or are different and each is $-CH_3$ or $-CH_2COO^-$; X^- is an anion and n is an integer), which process comprises reacting a compound of the formula:

(in which R^1 has the meaning defined above) in the form of a salt with an anion, with a compound of the formula:



(in which R^2 and R^3 have the meanings defined above) also in the form of a salt with an anion, in the presence of an acid binding agent, to yield a compound of the formula:



(in which Y is an anion and m is an integer); and subsequently, if desired, converting the anion Y to an anion X.