Et_3N was added dropwise with stirring. After 2 hr the solution was washed (dilute $NaHCO_3$, H_2O, saturated NaCl solution). After drying (Na_2SO_4), the solution was evaporated to give 19.5 g of a brown oil. This material was dissolved in C_6H_6 and chromatographed on 386 g of silica gel. The column was eluted with a gradient system: 5% EtOAc/C_6H_6–30% EtOAc/C_6H_6–EtOAc. This procedure gave 10.7 g of chromatographically pure material. Crystallization from C_6H_{14} afforded 8.8 g of a solid, mp 150–153°.

3β-Hydroxy-5-cholestene 4-(1-Aziridinyl)-3,5-dinitrobenzoate Ester (Method B, Table III).—To a solution of 8.21 g (31 mmoles) of 4-chloro-3,5-dinitrobenzoyl chloride in 100 ml of C_6H_6 was added 10 g (26 mmoles) of cholesterol and 10 ml of C_5H_5N. After stirring at room temperature for 2 hr, the solid material was removed by filtration and the filtrate was washed (dilute NaHCO$_3$, H_2O saturated NaCl). After drying the solution (Na_2SO_4), the liquid was evaporated to give 15.1 g of a crystalline solid. Crystallization from MeOH gave 13.8 g of a pale yellow solid, mp 170–173°. To a solution of 11.5 g of this material in 300 ml of DMF was added 10 ml of Et_3N and 2 ml of ethylenimine. This solution was stirred at room temperature for 20 min and diluted with 600 ml of EtOH. After cooling the solution to 0°, the crystalline precipitate was filtered off and dried to give 11.2 g of a yellow solid, mp 202–205°.

Estrone 4,6-Bis(1-aziridinyl)-*s*-triazin-2-yl Ether (Method D, Table III).—A mixture of 10 g (36 mmoles) of estrone, 7.31 g (37 mmoles) of 2-chloro-4,6-bis(1-aziridinyl)-*s*-triazine,[23] and 5.11 g (37 mmoles) of K_2CO_3 in 400 ml of dry Me_2CO was refluxed with stirring for 17 hr. After cooling, the reaction mixture was poured into 3 l. of H_2O with stirring. The resulting precipitate was filtered off and dried to give 16.2 g of a colorless powder. Crystallization from *i*-PrOH afforded 11.8 g of crystals, mp 156–160° dec. This material was recrystallized from *i*-PrOH to give 11.1 g, mp 170° dec.

3β-Acetoxy-16α-(1-aziridinyl)-5α-pregnane-12,20-dione (Method E, Table III).—3β-Acetoxy-5α-pregn-16-en-12,20-dione (10 g) was dissolved in 100 ml of ethylenimine and 2 ml of Et_3N was added. After standing at room temperature for 2.5 hr, the liquid was removed *in vacuo* and the residue was crystallized from petroleum ether (bp 30–60°) to give 9.9 g of crystals, mp 141–144°. Recrystallization from the same solvent gave 9.7 g, mp 145–147°.

17α-(1-Aziridinylmethyl)-3β,17β-dihydroxy-5-androstene (Method C, Table III).—A solution of 19.86 g of 3β-hydroxyspiro-17β-oxiranylandrost-5-ene[24] in 400 ml of ethylenimine containing a catalytic amount of MeONa was maintained at 100 ± 5° in a sealed pressure bomb for 10 hr. The solution was cooled to room temperature and the liquid was evaporated. The residue was dissolved in $CHCl_3$ and this solution was washed (H_2O) and dried (Na_2SO_4). Evaporation of the liquid afforded 21.9 g of a solid. Crystallization of this material gave 14.46 g of chromatographically pure material.

3β-Hydroxy-5-cholestene Aziridinylacetate.—Cholesterol chloroacetate (10 g, 22 mmoles) was combined with 50 ml of dry C_6H_6 and 100 ml of Et_3N. To this solution was added 10 ml (238 mmoles) of ethylenimine. The reaction mixture was stirred at room temperature for 24 hr, whereupon 5 ml more ethylenimine was introduced. Stirring was continued for 48 hr. The reaction mixture was then diluted with 500 ml of 1% Et_3N in C_6H_{14} and filtered through Supercel. Evaporation of the solvent afforded 10 g of crude product. This residue was combined with 8 g of crude product from two previous reactions. The total crude product was subjected to reversed-phase partition chromatography using a system composed of Et_3N–C_7H_{16}–MeCN (1:25:100). The less polar phase (2250 ml) was supported on a 10 × 95 cm column of 2500 g of silylated Supercel, and the column was eluted with 30 l. of polar phase. Fractions containing pure product were evaporated at room temperature and 0.5 mm to give 11.3 g (57%) of analytical quality cholesterol β-aziridinylacetate. The compound had no true melting point but rather softened to a gel in an evacuated capillary at 90–97°.

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(23) F. C. Schaefer, J. T. Geoghegan, and H. W. Kaiser, *J. Am. Chem. Soc.*, **77**, 5918 (1955).

(24) D. Rosenthal, L. Burger, and M. E. Wall, to be published.

Fluorescent Alkylating Agents. 1-(β-Chloroethyl)bisbenzimidazoles[1]

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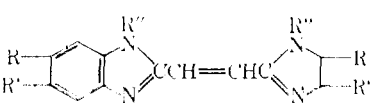
cis- and *trans*-1-(β-chloroethyl)bisbenzimidazoles have been synthesized as fluorescent alkylating agents. Preliminary *in vivo* study with HeLa cells shows that such compounds can be useful to demonstrate the intranuclear alkylation in dividing cells.

In spite of the numerous studies that suggested the reaction of alkylating agents with DNA or RNA or other nuclear material of a tumor cell, no direct *in vivo* evidence has ever been offered to demonstrate the uptake of an alkylating agent inside a cancer cell.

Fluorescent alkylating agents should be especially useful for such a purpose because fluorescence could be detected visually after reaction and measured fluorometrically. A histochemical fluorescent alkylating agent should possess a high degree of fluorescence so that the alkylating site would be suitably sensitive. Preferably, in *in vivo* experiments, the fluorescence should not be masked by the autofluorescence of the surrounding normal cells. The alkylating agents should not only have a good degree of substantivity but, for *in vivo* work, they should also not be easily metabolized. Since the incorporation of a benzimidazole ring into a nitrogen mustard had previously resulted in a clinically palliative alkylating agent,[2] the *cis*- and *trans*-1-(β-chloroethyl)bisbenzimidazoles (**1**) were there-



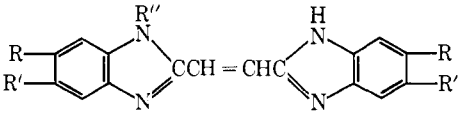
1, R = H or CH_3; R' = H or CH_3; R'' = CH_2CH_2Cl

(1) This work was supported by U. S. Public Health Grant CA-07339 from the National Cancer Institute, National Institutes of Health, Bethesda, Md. Presented in part at the 1st Mid-Atlantic Regional Meeting of the American Chemical Society, Philadelphia, Pa., Feb 1966.

(2) J. E. Ultmann, H. G. Thompson, E. Hirshberg, J. Zaidenweber, and A. Gellhorn, *Cancer Res.*, **19**, 719 (1959).

TABLE I

α,β-DI(2-BENZIMIDAZOLYL)ETHYLENE DERIVATIVES



No.	Abbr.[a]	Isomer	R	R′	R″	Mp, °C[b]	Yield, %	Formula	Analyses
2a	DBE	*cis*	H	H	H	405–407	15.5	$C_{16}H_{12}N_4$	C, H, N
b		*trans*				437–439	93.5	$C_{16}H_{12}N_4$	N
3a	Me-DBE	*cis*	Me	H	H	>500	7.9	$C_{18}H_{16}N_4$	C, H, N
b		*trans*				>500	44.3	$C_{18}H_{16}N_4$	N
4a	Me_2-DBE	*cis*	Me	Me	H	308–310	6.7	$C_{20}H_{20}N_4 \cdot EtOH$	C, H, N
b		*trans*				343–345	75.5	$C_{20}H_{20}N_4$	N
5a	OH-DBE	*cis*	H	H	CH_2CH_2OH	268–270	14.9	$C_{18}H_{16}N_4O$	C, H
b		*trans*				278–280	9.1	$C_{18}H_{16}N_4O$	N
6a	OH-Me-DBE	*cis*	Me	H	CH_2CH_2OH	285–286	14.2	$C_{20}H_{20}N_4O$	C, H
b		*trans*				298–300	26.7	$C_{20}H_{20}N_4O$	C, H, N
7a	OH-Me_2DBE	*cis*	Me	Me	CH_2CH_2OH	295–297	7.2	$C_{22}H_{24}N_4O$	C, H, N
b		*trans*				303–305	7.7	$C_{22}H_{24}N_4O$	C, H, N
8a	Cl-DBE	*cis*	H	H	CH_2CH_2Cl	205–207	28.3	$C_{18}H_{15}ClN_4 \cdot EtOH$	C, H
b		*trans*				228–230	7.7	$C_{18}H_{15}ClN_4$	N
9a	Cl-Me-DBE	*cis*	Me	H	CH_2CH_2Cl	144–146	20.3	$C_{20}H_{19}ClN_4$	N
b		*trans*				175–177	12.0	$C_{20}H_{19}ClN_4$	N
10a	Cl-Me_2-DBE		Me	CH_3	CH_2CH_2Cl				
b		*trans*				300–302	<1.0	$C_{22}H_{23}ClN_4$	N

[a] See full name in Experimental Section. [b] Melting points were obtained on the free base in each case. The melting points are uncorrected and were obtained on a Mel-Temp lab device.

fore considered to be potentially useful fluorescent alkylating agents.

In this type of compound, the two haloalkyl groups are not on the same N atom and, therefore, may be active independent of each other in contrast to the N mustards of the HN_2 type. Electron-donating CH_3 groups in the benzene rings should be expected to enhance the biological activity by increasing the electron density of the N atom and the reactivity of the halogen atoms. The present paper reports the synthesis of these compounds, the study of their fluorescence behavior, and a preliminary study of the interaction of one of them with HeLa cells *in vivo* as a demonstration of the usefulness of such methods.

The synthetic scheme is illustrated in Chart I. The reaction of an *o*-phenylenediamine with maleic acid gave predominantly *cis*-bisbenzimidazole (**2a**, **3a**, **4a**) (DBE). If malic acid was used in the presence of polyphosphoric acid, mainly *trans*-bisbenzimidazole (**2b**) resulted. The resulting bisbenzimidazole was then treated with ethylene chlorohydrin in the presence of NaOH to yield the hydroxyethylbisbenzimidazoles (**5**–**7**). The hydrochlorides of the corresponding chloroethylbisbenzimidazole could then be converted to the corresponding free bases (**8**–**10**) with $NaHCO_3$. The compounds thus prepared are listed in Table I.

The *trans* isomer of most *cis*-*trans* pairs has a higher melting point than the *cis* isomer. This difference may be due in part to a facile intermolecular H bonding in the *trans* structure, as this later found support in our ir study.

In the preparation of DBE (**2**), Me-DBE (**3**), and Me_2-DBE (**4**), the yield was higher for the *trans* isomer than the *cis* isomer. An examination of a molecular model of the *cis* isomer suggested that there is steric crowding due to the presence of benzimidazolyl groups on the same side of the double bond. As a result, α-(2-benzimidazolyl)-β-(aniline-2-carboxamido)-ethylene was found to be present in the reaction mixture as the major by-product.

Some of the bisbenzimidazoles were submitted for antitumor screening at CCNSC. DBE showed no antitumor activity for L1210 at a nontoxic dose. All other compounds showed some activity in 9-KB cells, but no structure–activity relationship could be derived because of insufficient data.

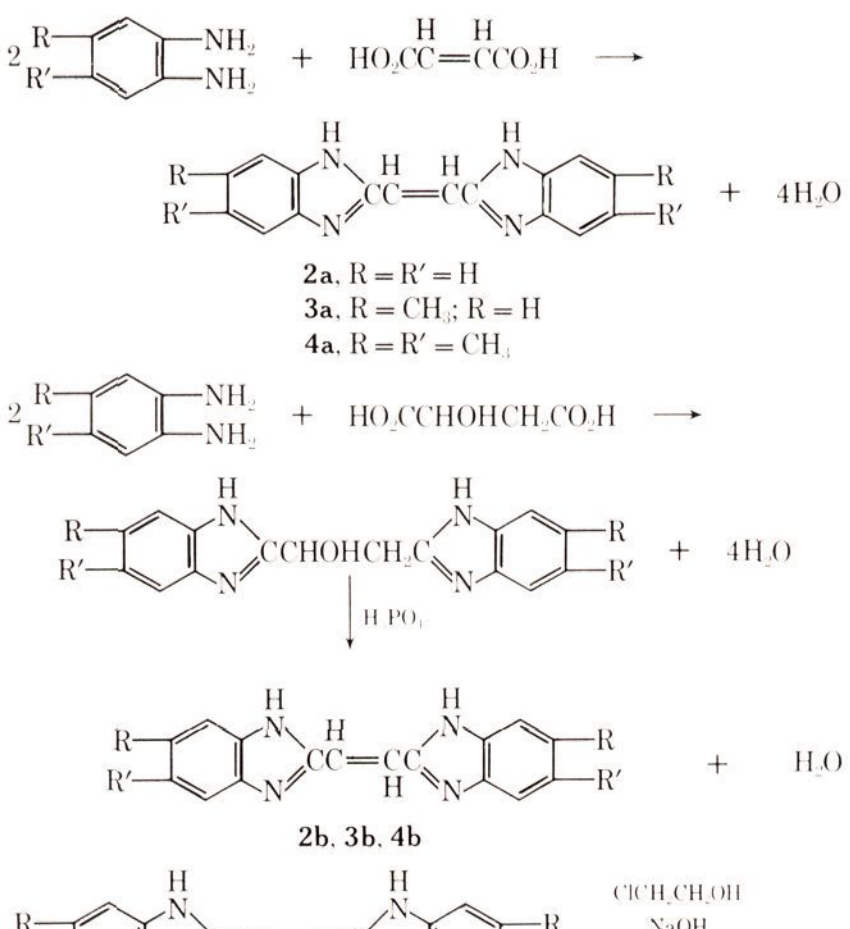
The preparation of the dihaloalkylbisbenzimidazoles was facilitated by the reaction of a bisbenzimidazole with $NaNH_2$ to form the 2Na salt, followed by reaction with ethylene chlorohydrin to form the dihydroxyethyl derivative. This dihydroxy compound was then treated with $SOCl_2$ to form the dichloroethylbisbenzimidazole. This procedure as applied to bisbenzimidazoles is still in the investigative stage.[3]

In most cases, the *trans* isomer shows higher fluorescence maxima and more intense fluorescence than the corresponding *cis* isomer. In general, the introduction of alkyl or substituted-alkyl groups shifts the fluorescence maximum to longer wavelengths.

In preliminary work on the ability of *trans*-Cl-Me-DBE (**9b**) to alkylate HeLa cells *in vivo*, alkylation appeared to have taken place when the sites of alkylation could be seen by the fluorescence of the alkylating agent. In different stages of alkylation fluorescence appeared at first (2–6 hr) in the cytoplasm and later on (8–24 hr) in the nucleus. The *cis* compound was less efficient showing relatively low fluorescence and ease of quenching during microscopic observation. While extensive nuclear debris containing fluorescent material could be seen, there were observed numerous colonies of fluorescent-nuclei-containing HeLa cells that suggested that these cells were alive during the fixation step with formaldehyde. Also, these fluo-

(3) T. Seki, M. Sasazima, and Y. Watanabe, *Yakugaku Zasshi*, **85**, 962 (1956).

Chart I



rescent nuclei suggested strongly that they could be of the nucleolar type and, therefore, related to RNA as well as DNA alkylation when the same cells are visualized under fluorescence phase microscopy.

Experimental Section

cis-**α,β-Di(2-benzimidazolyl)ethylene (Ia, DBE).**—The preparation of **2a** was modified from a procedure reported in the patent literature.[4] All other *cis* derivatives were prepared similarly and the yield and physical constants are given in Table I.

Maleic acid (1.16 g, 0.01 mole) was mixed with 6.1 g (0.057 mole) of *o*-phenylenediamine. The mixture was heated under N_2 with stirring for 3 hr at 150–160°. The mixture was then allowed to come to room temperature. The resulting solidified mass was extracted with EtOH (93 ml). The remaining residue was dissolved in 1050 ml of 4 *N* HCl, and the solution was boiled with charcoal for 10 min. The charcoal was removed by filtration, and the filtrate was cooled and made alkaline with concentrated NH_4OH. A precipitate of DBE formed which after cooling was collected, washed (EtOH), and allowed to dry in the air. It had mp 405–407°, yield 0.68 g (26.0%). *Anal.* ($C_{16}H_{12}N_4$) C, H, N. This material could be converted to the HCl salt and recrystallized from 4 *N* HCl. Other bisbenzimidazole hydrochlorides were prepared in the same way. In most cases the hydrochlorides had melting points higher than 300°.

(4) Ciba Ltd., Swiss Patent 240,109 (March 16, 1946) (addition to Swiss Patent 238,148 (1946)).

The EtOH extract of the solidified mass resulting from the reaction was concentrated to dryness by gentle heating. The residue was stirred at room temperature for 1 hr with 125 ml of 5% (w/w) aqueous $NaHCO_3$ and the mixture was allowed to stand at room temperature overnight. The undissolved by-product was collected, dissolved in 4 *N* HCl, and reprecipitated by addition of solid $NaHCO_3$ to the solution. An analytical sample was prepared by recrystallization (EtOH–Et_2O). The elemental analysis was consistent with the formation of the dihydrochloride of α-(2-benzimidazolyl)-β-(aniline-2-carboxamido)-ethylene dihydrochloride. *Anal.* ($C_{16}H_{14}N_4O \cdot 2HCl$) C, H, N. Addition of solid $NaHCO_3$ to a 4 *N* HCl solution of this material apparently resulted in the salting out of this dihydrochloride rather than conversion to the free base. This by-product was moderately soluble in H_2O at pH ~6. Cl^- was shown by a positive $AgNO_3$ test. The recovery was 1.0 g (28.5%), mp 208–210°. The aqueous filtrate from the work-up of this material was acidified with 4 *N* HCl and the resulting solution was concentrated to dryness under vacuum with gentle heat. The residue was recrystallized from EtOH–Et_2O to give recovered *o*-phenylenediamine dihydrochloride, mp 230–232°, and ir spectrum identical with an authentic sample.

α,β-Di(2-benzimidazolyl)ethylene (2b).—This reaction was based on a procedure for the preparation of DBE reported in the patent literature.[5] Other *trans*-bisbenzimidazoles were prepared according to the same general procedure.

Maleic acid (2.68 g, 0.02 mole) and *o*-phenylenediamine (4.32 g, 0.04 mole) were mixed with 40 g (0.41 mole) of polyphosphoric acid. The reaction mixture was heated with stirring under N_2 at 160° for 2.5 hr. The reaction mixture was then poured with stirring into 250 ml of cold H_2O and the pH of the resulting mixture was adjusted to 8 by the addition of 30% NaOH. A precipitate of DBE formed, which after cooling was collected by filtration, washed [H_2O, petroleum ether (bp 30–60°)], and allowed to dry in the air. It had mp 437–439°, yield 6.2 g (93.5%). This material was characterized as the monohydrochloride hydrate. *Anal.* ($C_{16}H_{13}N_4 \cdot 4H_2O \cdot HCl$) C, H.

cis-**α-(2-[1-(2-Hydroxyethyl)benzimidazolyl])-β-(2-benzimidazolyl)ethylene (2a, OH-DBE).**—This preparation was also modified from a procedure for the preparation of OH-DBE reported by Ackermann and Meyer.[6] Other *cis*- and *trans*-hydroxyethylbisbenzimidazoles were prepared in a similar way.

DBE (0.72 g, 0.003 mole, from maleic acid) was suspended in 14 ml of EtOH and a solution of 0.4 g (0.01 mole) of NaOH in 2 ml of H_2O was added to the suspension. The resulting mixture was heated to boiling, and 0.4 ml (0.006 mole) of ethylene chlorohydrin was added dropwise over 75 min to the boiling mixture. The reaction mixture was then refluxed for 3 hr and cooled, and the undissolved material was removed. The filtrate was poured with stirring into 150 ml of cold H_2O, and then a

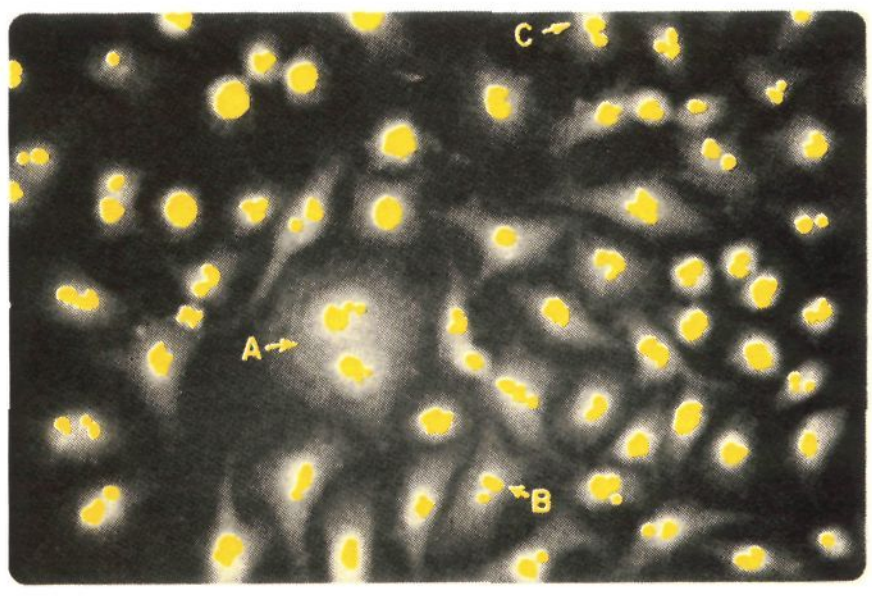


Figure 1.—Fluorescent nuclei in Hela cells 24 hr after inoculation at a concentration of 10^{-5} *M* Cl-Me-DBE (*trans*)/ml, fixed in formal–calcium for 15 min; magnification 500 ×: (A) nucleolar and chromatin fluorescence in a polynuclear cell; (B) a metaphase cell; (C) an anaphase cell, as checked under the phase contrast microscope.

(5) Ciba Ltd., British Patent 861,431 (Feb 22, 1961).

(6) F. Ackermann and J. Meyer (to Ciba Ltd.), U. S. Patent 2,604,454 (July 22, 1952).

TABLE II

UV AND VISIBLE SPECTRA OF BISBENZIMIDAZOLES[a]

No.	λ_{max}, mμ (ε)
2a	241.3 (39,300), 272.5 (51,200), 278.8 (62,400), 340.0 (755), 360.0 (1080), 377.5 (216)
b	225.0 (sh) (16,100), 245.0 (11,300), 275.0 (12,300), 280.0 (13,200), 345.0 (32,100), 360.0 (38,300) 380.0 (26,700)
3a	222.5 (sh) (29,600), 255.0 (sh) (35,100), 262.5 (40,900) 348.8 (40,400), 367.5 (50,500), 387.5 (40,400), 417.5 (11,900)
b	225.0 (sh) (2800), 272.5 (8300), 350.0 (3340), 367.5 (4630), 387.5 (3340)
4a	255.0 (sh) (9700), 270.0 (9700), 278.8 (9700), 282.5 (9700), 288.7 (10,400), 335.0 (6050), 355.0 (6470), 375.0 (4840), 397.5 (3550), 427.5 (755)
b	225.0 (sh) (11,600), 357.5 (22,600), 375.0 (30,100), 395.0 (21,700)
5a	245.0 (23,500), 275.0 (15,600), 282.5 (18,700), 355.0 (41,800), 370.0 (43,000), 392.5 (sh) (25,600)
b	245.0 (sh) (21,500), 275.0 (sh) (15,600), 282.5 (18,300), 355.0 (41,000), 370.0 (39,300), 392.5 (sh) (22,400)
6a	232.5 (sh) (22,600), 252.5 (sh) (14,500), 365.0 (40,900), 380.0 (43,000), 387.5 (sh) (27,700)
b	225.0 (sh) (30,400), 250.0 (sh) (14,400), 370.0 (73,000), 390.0 (51,500)
7a	245.0 (sh) (19,800), 282.5 (17,800), 288.7 (19,800), 362.5 (sh) (29,500), 380.0 (34,200), 402.5 (sh) (22,400)
b	230.0 (sh) (30,500), 245.0 (sh) (20,700), 282.5 (sh) (12,800), 290.0 (14,300), 365.0 (sh) (53,200), 380.0 (61,400), 402.5 (sh) (41,300)
8a	243.8 (19,800), 273.8 (15,900), 280.0 (17,700), 348.8 (28,000), 366.3 (31,400), 385.0 (sh) (19,500)
b	227.5 (sh) (18,000), 245.0 (sh) (12,800), 275.0 (sh) (7000), 282.5 (7850), 350.0 (35,700), 365.0 (39,800), 387.5 (sh) (25,600)
9a	217.5 (430), 230.0 (sh) (755), 270.0 (2790)
b	227.5 (sh) (46,300), 250.0 (sh) (27,600), 272.5 (sh) (16,600) 290.0 (19,900), 372.5 (>100,000), 390.0 (sh) (73,100)
10a	...
b	227.5 (sh) (12,300), 290.0 (sh) (4520), 377.5 (27,700), 395.0 (sh) (21,100)

[a] All spectra were obtained for 9.3×10^{-6} *M* solutions of bisbenzimidazole in EtOH on a Beckman DB-G grating spectrophotometer.

precipitate of crude OH-DBE formed which was collected. This material was dissolved in EtOH (25 ml), the resulting solution was decolorized with charcoal, and the filtrate was allowed to stand at room temperature. The precipitate of IVa was collected in two fractions by filtration and allowed to dry in the air. It had mp 263–267°, yield 0.18 g (15%). Further recrystallization from EtOH gave material melting at 268–270°. All compounds in this series tenaciously held onto solvent of crystallization.

***cis*-α-(2-[1-(2-Chloroethyl)benzimidazolyl])-β-(2-benzimidazolyl)ethylene (8a, Cl-DBE).**—The preparation of **7a** was adapted from a procedure by Schmutz and Kunzle[7] for the preparation of 2-chloromethyl-3-(β-chloroethyl)benzimidazole from the corresponding hydroxy derivative. All other chloroethyl derivatives were prepared similarly (Table I).

Compound **5a** (0.1 g, 2.3×10^{-4} mole) was stirred with 20 ml of $CHCl_3$ with protection from moisture. A solution of 2 ml (3.32 g, 0.028 mole) of $SOCl_2$ in 10 ml of $CHCl_3$ was then added slowly with stirring. Stirring was continued and the mixture was refluxed for 3 hr. Upon cooling a large excess of anhydrous Et_2O was added. A flocculent yellow precipitate of **8a** formed as a hydrochloride which was collected, washed (Et_2O), and stored in a dessicator over $CaSO_4$. The yield was 0.09 g (96.8%), mp 165–175°. The hydrochloride was converted to its free base in H_2O with saturated $NaHCO_3$, mp 197–200° after air drying. Recrystallization from EtOH gave small yellow crystals melting at 205–207°, and again containing 1 mole of EtOH as solvent of crystallization.

(7) G. Schmutz and F. Kunzle, *Helv. Chim. Acta*, **39**, 1144 (1956).

Uv and Visible Spectra Study.—Uv and visible spectra of the bisbenzimidazoles were obtained on a Beckman DB-G grating spectrophotometer as 9.3×10^{-6} *M* solutions in EtOH (Table II).

One would expect the *trans* isomer to have a greater λ_{max} and corresponding ε than the *cis* compound, particularly for the longer wavelength bands. This pattern is borne out for most compounds by their uv and visible spectra, except Me-DBE (**3a,b**) and OH-DBE (**5a,b**).[8]

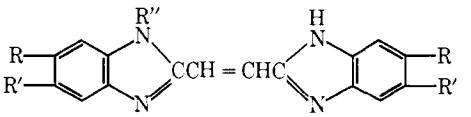
Some of the bisbenzimidazoles showed a doublet absorption maxima around 280 mμ characteristic of benzimidazoles, 5(6)-methylbenzimidazole, or 5,6-dimethylbenzimidazole.[9] In these cases the *cis* compound often showed more intense absorption than the *trans* compound for these absorption bands.

Ir Spectra Study.—Ir spectra for the bisbenzimidazoles were obtained in KBr pellets using a Perkin-Elmer 137 NaCl spectrophotometer. The ir data gave contributing evidence to the assignment of *cis* and *trans* forms. The *trans* compounds all had a stronger absorption at 970–960 cm^{-1} (C–H out-of-plane deformation) compared to the *cis* isomer.

Fluorescence Spectra Study.—Fluorescence spectra of the bisbenzimidazoles were determined on an Aminco-Bowman spectrophotofluorometer as 4.7×10^{-6} *M* solutions in EtOH (Table III).

TABLE III

FLUORESCENCE SPECTRA OF BISBENZIMIDAZOLES[a]



No.	Config	Excitation max, mμ (rel intensity)	Fluorescence max, mμ (rel intensity)
2a	*cis*	370 (0.300)	423 (0.254)
b	*trans*	353 (8.9)	425 (13.4)
3a	*cis*	377 (9.15)	435 (8.25)
b	*trans*	365 (1.4)[b]	435 (1.67)[b]
4a	*cis*	385 (1.79)	443 (1.56)
b	*trans*	383 (7.75)	445 (7.00)
5a	*cis*	380 (7.65)	443 (7.25)
b	*trans*	380 (14.4)	445 (13.5)
6a	*cis*	390 (8.80)	455 (8.05)
b	*trans*	378 (17.5)	440 (16.9)
7a	*cis*	385 (8.40)	443 (9.00)
b	*trans*	393 (15.1)	455 (15.1)
8a	*cis*	377 (5.18)	435 (4.56)
b	*trans*	375 (8.25)	435 (7.98)
9a	*cis*	377 (0.111)	440 (0.118)
b	*trans*	370 (29.2)	443 (28.0)
10a	...	...	...
b	*trans*	385 (6.58)	450 (6.57)

[a] All spectra were obtained for 4.7×10^{-6} *M* solutions of bisbenzimidazole in EtOH on an Aminco-Bowman spectrophotofluorometer. [b] The anomaly here may be due to 5′- and 6′-dimethyl isomers.

Titration of Bisbenzimidazoles.—This procedure was based on a method by Fritz and Hammond.[10] $HClO_4$ (70–72%) (8.5 ml) was mixed with 200 ml of AcOH and 20 ml of Ac_2O. The solution was allowed to stand overnight to permit complete reaction of Ac_2O with the H_2O present. This solution was then diluted to 1 l. with AcOH to make a solution ~0.1 *N* with respect to $HClO_4$. Of this solution 10 ml was diluted to 1 l. with AcOH to make a

(8) For these bisbenzimidazoles, isomerization must have taken place in solution readily. The resonance hydrid of the ground state may have contributions from various resonance forms. Resonance forms of the latter type have a single bond at the place where isomerization takes place and contributions from these forms should decrease the barrier to rotation around this bond and increase the possibility for isomerization. A similar explanation has been set forth to explain anomalous results in connection with some *cis* and *trans* stilbene compounds [M. Calvin and R. E. Buckles, *J. Am. Chem. Soc.*, **62**, 3324 (1940)].

(9) K. Hofmann, "The Chemistry of Heterocyclic Compounds: Imidazole and its Derivatives," Part I, Interscience Publishers, Inc., New York, N. Y., 1953, p 253.

(10) J. S. Fritz and G. S. Hammond, "Quantitative Organic Analysis," John Wiley & Sons, Inc., New York, N. Y., 1957, pp 265–266.

solution which was approximately 0.001 *N* with respect to $HClO_4$. It was standardized by titrating it against 0.00102 g (5.0×10^{-6} mole of potassium acid phthalate in 50 ml of glacial AcOH) using a Corning Model 12 pH meter equipped with glass and calomel electrodes. Readings on the + mV scale were recorded for the corresponding milliliters of titrant. Milliliters of titrant were then plotted against + mV readings, and the end point was determined graphically. The standardized $HClO_4$ solution was then used for similar potentiometric titrations of bisbenzimidazoles (5.0×10^{-6} mole of bisbenzimidazole in 50 ml of glacial AcOH). The titration was carried out until the first break in the curve was obtained and this value corresponded to the protonation of one of the N atoms in the molecule. Because of the ease of solvent trapping in these compounds, titration with this method is considered the most reliable method for purity determination.

HeLa Cell Alkylation Study.—HeLa cells were harvested in Leighton tubes containing cover slips and inoculated with *trans*-Cl-Me-DBE. At a concentration of 10^{-5} to 10^{-6} *M* ml, the effect could clearly be seen *in vivo*. At 2–8 hr the nuclear membrane started to show fluorescence as well as about 15% of the nuclei of the cell population (variation depending on original culture), and, at the end of 24 hr, numerous cells appeared fluorescent in their nuclei (Figure 1). Thus, it was felt that in spite of the low solubility of the compound, the incorporation of a fluorescent alkylating agent into the nucleus was found to be possible. Efforts are being made to correlate fluorescence with various mitotic phases in synchronous population. Our present contention is satisfied with the knowledge that *in vivo* alkylation of nucleus, indeed, occurred with a fluorescent-labeling alkylating agent.

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Derivatives of Fluorene. XXX.[1] Rearrangement and Antitumor Activities of Some 9-Oxofluorene Oximes. 6(5H)-Phenanthridinones. I

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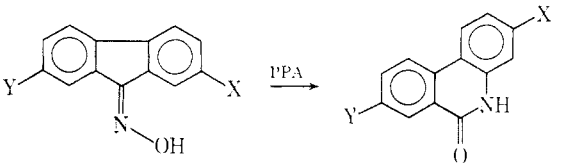
Rearrangement of 9-oxofluorene oximes in polyphosphoric acid (PPA) to the corresponding 6(5H)-phenanthridinones is described. Reaction of 1-iodo- and 1-nitro-9-oxofluorene oxime with PPA gave, instead of the expected phenanthridinones, the corresponding 9-oxofluorenes. Results of screening for antitumor activities are presented.

Because interesting antitumor activity was shown earlier[2] by a number of polyhalogenated fluorene derivatives, we have prepared a number of structurally related compounds with altered properties, *e.g.*, increased hydrophilicity, which might enhance the biological effects of these compounds. One such series is a group of oximes of 9-oxofluorenes (Table I).[3] Several of these have shown activity against Walker carcinosarcoma 256 (see Table II). A further reason for our interest in these oximes is that rearrangement to phenanthridinones (Table I) gives a heterocyclic system which has had few derivatives screened for antitumor activity. We are particularly interested in polyhalogenated phenanthridinones analogous to the active compounds in the fluorene series.[2] This paper is the beginning of such a study.

The oximes were prepared in DMSO, by an improved procedure,[3] or in the conventional way by treating the 9-oxofluorene with 2 equiv of hydroxylamine hydrochloride in refluxing 70% EtOH. The rearrangement of the 9-oxofluorene oximes was carried out in polyphosphoric acid[4] (PPA) at elevated temperatures.

Although the oxime of 3-nitrofluorenone in PCl_5–$POCl_3$ rearranged to a single compound, 2-nitrophenanthridinone,[5] monosubstituted 9-oxofluorene oximes, in general, rearrange to a mixture of the two isomers, difficult to separate. Even in PPA such mixtures are to be expected; however, in our work, 2-nitrofluorenone oxime gave a fair yield of only one product, 3-nitrophenanthridinone.

A series of 2,7-disubstituted fluorenone oximes, with both substituents the same, gave good yields of 3,8-disubstituted phenanthridinones (eq 1) when they were heated for 15 min at temperatures above 180°. It was reported earlier[6] that fluorenone oximes did not rearrange at temperatures of 100–150°, effective for many oximes.



(1)

Ia. X = NO_2; Y = H
b. X = Y = F
c. X = Y = Cl
d. X = Y = Br
e. X = Y = NO_2

In spite of the two paths followed in the Beckmann rearrangement of many of these monosubstituted oximes, it was hoped that a bulky substituent, such as iodo or nitro, at the 1 position of the fluorene nucleus would lead to a single product, hopefully a 4-substituted 6(5H)-phenanthridinone. However, the only identifiable product obtained from each of these reactions was the corresponding 1-substituted 9-oxofluorene (eq 2).

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(2) H.-L. Pan and T. L. Fletcher, *J. Med. Chem.*, **7**, 31 (1964); H.-L. Pan and T. L. Fletcher, *ibid.*, **8**, 491 (1965).

(3) H.-L. Pan and T. L. Fletcher, *Chem. Ind.* (London), 240 (1969), paper XXIX in this series.

(4) E. C. Horning and V. L. Stromberg, *J. Am. Chem. Soc.*, **74**, 2680 (1952).

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