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(57) Abstract

The present invention is directed to bistriazene compounds, pharmaceutical compositions containing effective anti-cancer amounts of these compounds, a method for treating cancer comprising administering to affected subjects an anti-cancer effective amount of a bistriazene compound, and the use of bistriazene compounds as cross-linking reagents applicable to the synthesis and manipulation of polymeric macromolecules.

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BISTRIAZENES AS CHEMOTHERAPEUTIC AGENTS

This application is a continuation-in-part of application Serial No. 07/527,915 filed on May 24, 1990, "BISTRIAZENES AS CHEMOTHERAPEUTIC AGENTS", the entire contents of which are hereby incorporated by reference.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to the use of bistriazene compounds as chemotherapeutic agents useful in the treatment of various cancers. As such, these compounds find wide utility in both human and veterinary medicine. The invention also relates to the use of these compounds as crosslinking reagents useful in a wide variety of laboratory and chemical applications involving the synthesis and manipulation of polymeric macromolecules.

Description of Related Art

A number of chemotherapeutic agents exist which act as alkylating agents capable of forming covalent linkages with a variety of substances, including phosphate groups in DNA. Alkylation of bases in DNA often leads to gene miscoding, serious damage to the DNA molecule, and/or major disruption in nucleic acid function, and results in the inhibition of a wide range of other cellular functions. These agents act by forming lethal crosslinks in nucleic acid molecules, and can often shrink tumors in a matter of days after intravenous administration. Among these compounds are 2-chloroethyl-nitrosoureas such as bis(2-chloroethyl)nitrosourea (BCNU), mitomycin, cyclophosphamide (cytoxan), and ifosphamide. These agents are themselves potentially mutagenic, teratogenic, and carcinogenic, and their anti-neoplastic activity is exerted throughout the cell cycle, i.e., toxicity is cell cycle independent.

Vaughan et al. (1984) Jour. Med. Chem. 27:357-63 have reported the formation of a certain bistriazene as a by-product in the preparation of other triazenes. This bistriazene is chemically and structurally different from those of the present invention, and was not tested for antitumor activity. Furthermore, this bistriazene differs from those of the present invention in that it would require two-fold metabolic activation to release the same alkylating moiety, and is susceptible to hydrolysis, thereby releasing monotriazenes.

The use of the bistriazene compounds of the instant invention as chemotherapeutic and crosslinking agents has yet to be reported.

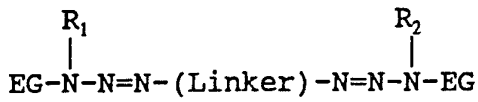
SUMMARY OF THE INVENTION

The bistriazene compounds of the present invention are novel alkylating agents which are structurally similar to polyamines such as spermine and spermidine, which interact with DNA. Most currently employed chemotherapeutic alkylating agents interact covalently or noncovalently with the target DNA, after which a crosslinking reaction may occur. The bistriazene compounds of the present invention differ from any known chemotherapeutic agents in that their chemical structure allows them to interact with the DNA molecule while maintaining their chemical integrity. This interaction depends on the formation of multiple hydrogen bonds with the DNA, and in this manner they appear to mimic natural polyamines which normally interact with DNA. In fact, it is possible that due to the structural similarity of the bistriazenes to some of the polyamines, the bistriazenes may occupy the same sites in DNA as the polyamines themselves. Subsequent to this binding, the bistriazenes decompose on the surface of the DNA, releasing the "Linker" in the form of a bisdiazonium ion. This highly reactive substance covalently interacts with the DNA, causing multiple double strand breaks and interstrand crosslinks. As the bisdiazonium ion can be made to vary in

its properties by structural modification of the Linker in the bistriazene molecule, the reactivity of the entire molecule can be modulated by appropriate chemical modification. Thus, it appears that bistriazenes may interact with DNA in a polyamine-like fashion, subsequently breaking down to form crosslinking agents which result in the formation of crosslinks lethal to cells. The use of the bistriazene compounds of the present invention as chemotherapeutic drugs therefore confers great specificity of drug interaction with DNA, and because the reactive diazonium ions are formed on the surface of the DNA, delivers a much higher effective dose of the ultimate cytotoxic agent per molecule of administered compound than for simple monodentate drugs. This feature achieves the advantage that the dosage of such bistriazene-based drugs administered will be low in comparison to that of other conventional chemotherapeutic alkylating agents. The combination of specificity and low effective dose portends bistriazene-based anti-cancer drugs with much lower systemic toxicities than those currently in use.

Thus, the bistriazene compounds of the present invention represent an entirely novel class of bidentate, chemotherapeutic alkylating agents with greater specificity and lower toxicicity as compared to present treatments.

Accordingly, it is an object of the present invention to provide a bistriazene compound, or a physiologicaly acceptable salt thereof, of the formula:



wherein

the Linker is selected from the group consisting of

, where n=1-5, and R_3 is selected from the group consisting of hydrogen and a C_1-C_5 n-alkyl,

$-(CH_2)_n-\underset{\displaystyle R_4}{\overset{|}{N}}-(CH_2)_m-\underset{\displaystyle R_5}{\overset{|}{N}}-(CH_2)_n-$, where n=1-5, m=1-5, and R_4 and R_5 each = C_1-C_5 n-alkyl,

$-(CH_2)_n-O-(CH_2)_n-$, where n=1-5,

$-(CH_2)_n-S-(CH_2)_n-$, where n=1-5,

$-(CH_2)_n-Se-(CH_2)_n-$, where n=1-5,

$-(CH_2)_n-\underset{\displaystyle O}{\overset{\|}{S}}-(CH_2)_n-$, where n=1-5, and

$-(CH_2)_n-SO_2-(CH_2)_n-$, where n=1-5;

EG is identical or independently selected from the group consisting of a phenyl group, a substituted phenyl group, an arylalkyl group, a substituted arylalkyl group, a condensed ring arylalkyl group, a heterocyclic group, an amine group, and a polyamine.

Another object of the present invention is to provide a pharmaceutical composition, comprising an anti-cancer effective amount of said bistriazene compound or physiologically acceptable salt thereof, and a pharmaceutically acceptable carrier.

Yet another object of the present invention is to provide a method for treating cancer in a mammal, including humans, which comprises administering to the subject an anti-cancer effective amount of said bistriazene compound or a physiologically acceptable salt thereof.

A further object of the present invention is to provide a method of inhibiting breakage or digestion of DNA or proteins, comprising treating said DNA or proteins with said bistriazene copound or physiologically acceptable salt thereof.

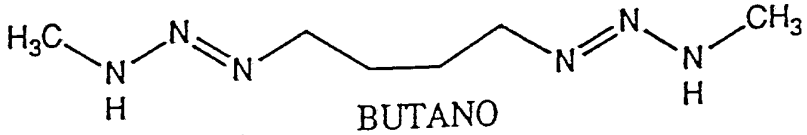
A still further object of the present invention is to provide a method of producing chemical polymers, comprising

treating their monomeric constituents with said bistriazene compound or physiologically acceptable salt thereof.

These objects and others are accomplished in accordance with the present invention by administering an anti-cancer effective amount of a pharmaceutical composition containing a bistriazene compound or a physiologically acceptable salt thereof. Representative bistriazene compounds useful in treating cancer include bis(methyltriazeno)-p-xylene, bis(methyltriazeno)-2-butene, bis(methyltriazeno)ethane, and other bistriazene derivatives which are anti-cancer agents, such as the following:

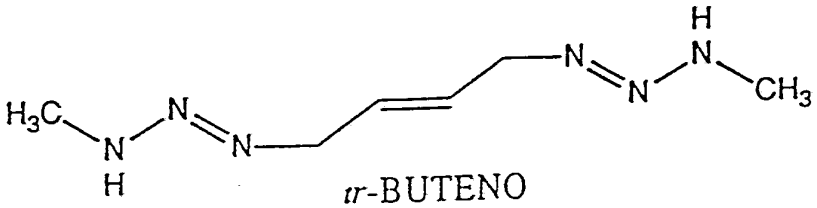
ETHANO

1,2-Bis(methyltriazeno)ethane



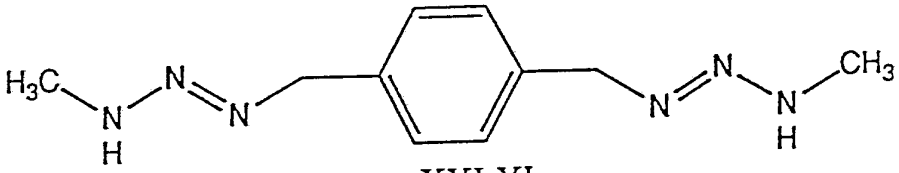
BUTANO

1,4-Bis(methyltriazeno)butane



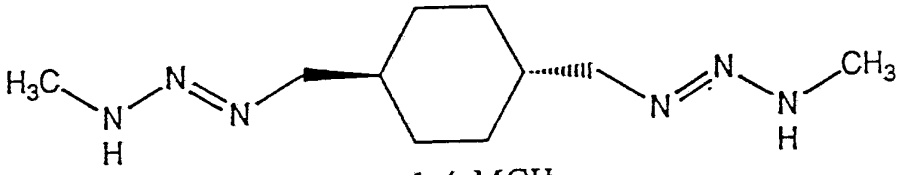
tr-BUTENO

1,4-Bis(methyltriazeno)-*trans*-2-butene



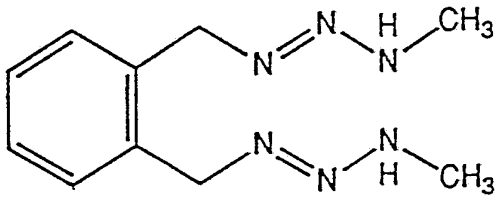
p-XYLYL

α,α'-Bis(methyltriazeno)-*p*-xylene



tr-1,4-MCH

trans-1,4-Bis(1,3-dimethyltriazeno)cyclohexane



o-XYLYL

α,α'-Bis(methyltriazeno)-*o*-xylene

The compounds of the present invention can be used for the treatment of human and animal cancers.

In addition to the use of bistriazene compounds for the therapeutic treatment of neoplastic disease, the use of these compounds as laboratory reagents is also envisioned as another object of the present invention. In the laboratory manipulation of macromolecules such as DNA and proteins, reagents are often employed which interact with the molecule of interest such that the molecule is:

1) Cut in a specific region;

2) Blocked from being enzymatically cut in a specific region;

3) Bound to another molecule with which it is loosely associated;

4) Bound to a matrix such as nitrocellulose or nylon to facilitate handling and probing;

5) Bound to a matrix such as a chromatography support as a ligand for affinity chromatography; or

6) Conjugated to unrelated macromolecules (e.g., toxins to antibodies, antibodies to enzymes, small molecules to oligonucleotide DNA probes, etc.).

Bistriazenes can be adapted for use in these and other laboratory manipulations of macromolecules.

If the bistriazene is modified such that the substituent groups afford a high degree of sequence recognition, then upon alkylation at a labile site, breakage of the DNA or protein backbone may occur (#1 above). Alternatively, alkylation at a stable site may block enzymatic digestion such as restriction enzyme digestion of DNA or protease digestion of proteins (#2 above).

Multifunctional chemical crosslinking agents are presently widely used in applications #3, #5, and #6 cited above. The use of bistriazene compounds in such applications is another object of the present invention.

It is further envisioned that bistriazene compounds will be employed as highly active chemical crosslinking

agents useful in immobilizing molecules such as RNA, DNA or proteins on nitrocellulose, nylon, or other similar membranes (application #4, above), thereby facilitating the handling and probing of these biopolymers on such membrane supports.

Yet another object of the present invention is to employ bistriazenes as crosslinking agents in the formation of chemical polymers from their monomeric constituents.

The bistriazene compounds of the present invention may be employed as chemical crosslinking agents in a manner similar to that of other well known crosslinking agents, as would be apparent to one of ordinary skill in the art.

Further scope of the applicability of the present invention will become apparent from detailed description and drawings provided below. However, it should be understood that the detailed description and specific examples, while indicating preferred embodiments of the invention are given by way of illustration only since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the survival in vitro of several human tumor cell lines exposed to various concentrations of bis(methyltriazeno)-p-xylene.

Figure 2 shows the survival in vitro of several human tumor cell lines exposed to various concentrations of bis(methyltriazeno)-2-butene.

Figure 3 shows the survival in vitro of several human tumor cell lines exposed to various concentration of bis (methyltriazeno)ethane.

Figure 4 shows the survival in vitro of several human tumor cell lines exposed to various concentrations of 5-(3,3-dimethyltriazeno)imidazole-4-carboxamide (DTIC).

In Figures 1-4, the abbreviations of the cell lines represent the following:

CXF, Colon Cancer Xenograft; GXF, Gastric; LXF, Lung: A adeno, L large cell, E epidermoid cell, S small cell; MAXF, Mammary Cancer Xenograft; MEXF Melanoma; PXF, Pleuramesothelioma; SXF, Sarcoma; TXF, Testicular; XF, miscellaneous Cancer Xenograft.

Figure 5 shows the results of the oligonucleotide crosslinking assay.

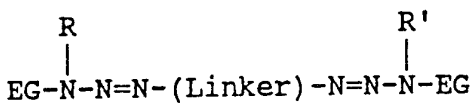
Figure 6 shows the results of the supercoiled plasmid DNA assay.

Figure 7 shows supercoiled plasmid pBR322 treated with various bistriazenes and triazenes indicated in the figure at the concentrations shown. The treated DNA was applied to agarose gels and electrophoresed. Bands were visualized by ethidium bromide staining. The bands of interest are SC = supercoiled plasmid, OC = open circular plasmid, linear=linearized plasmid. An absence of bands in a lane to which DNA had been applied indicates complete destruction of the DNA by the agent at that concentration. Formation of OC requires a single strand break; linearization requires a double strand break.

Figure 8 shows data for the bistriazenes and triazenes indicated, obtained in a manner similar to that in Figure 7. The applied concentrations of bis[2-(methyltriazeno)-ethyl]methylamine (3) were orders of magnitude lower than those for the other bistriazenes.

DETAILED DESCRIPTION OF THE INVENTION

As those of ordinary skill in the art will recognize, the basic bistriazene structure contains a number of elements which can be modified to affect the desired use of these compounds. These elements are indicated in the following structure:



The "Linker" moiety is involved in the structural

definition of the molecule and in crosslink formation. The Linker can be either an alkyl group, substituted alkyl (including, but not limited to, alkylamines, alkyl ethers and thioethers, haloalkyl, silanes, phosphines, alcohols, amines, etc.), of chain length 1-20, preferably 2-8. The Linker may also include aralkyl or substituted aralkyl (with modifications analogous to those for substituted alkyls), polycyclic aralkyl, heterocyclic aralkyl, and their substituted derivatives wherein the triazine moieties can be separated by 1-30 carbon atoms, preferably 4-12 carbon atoms. With regard to the "End Group" (EG), this moiety is crucial in modulating the reactivity of bistriazenes. The EGs may be identical or independently selected from groups comprising alkyl groups, substituted alkyl (including, but not limited to, alkylamines, alkyl ethers and thioethers, haloalkyl, silanes, phosphines, alcohols, amines, etc.), of chain length 1-20, preferably 1-6. The EG may also include aralkyl or substituted aralkyl (with modifications analogous to those for substituted alkyls), polycyclic aralkyl, aryl groups and heterocyclic groups of 2-40 non-hydrogen atoms, containing 1-6 rings, including nucleic acid bases and oligonucleotides.

The final substituent on the triazene moiety, i.e., R or R', is perhaps the most fungible, and may be added following assembly of the basic triazene moiety by methods described for simple dialkyltriazenes (R.H. Smith, Jr., et al., *J. Org. Chem.*, **1986**, *51*, 3751; R.H. Smith, Jr., et al., *J. Org. Chem.*, **1988**, *53*, 1467; D.H. Sieh, et al., *J. Am. Chem. Soc.*, **1980**, *102*, 3883; R.H. Smith, Jr. and C.J. Michejda, *Synthesis*, **1983**, 476). R or R' may be identical to EG or to one another, or may be independently selected from the groups comprising hydrogen, alkyl groups, substituted alkyl (including, but not limited to, alkylamines, alkyl ethers and thioethers, haloalkyl, silanes, phosphines, alcohols, amines, etc.) of chain length 1-20, preferably 1-6. R or R' may also include

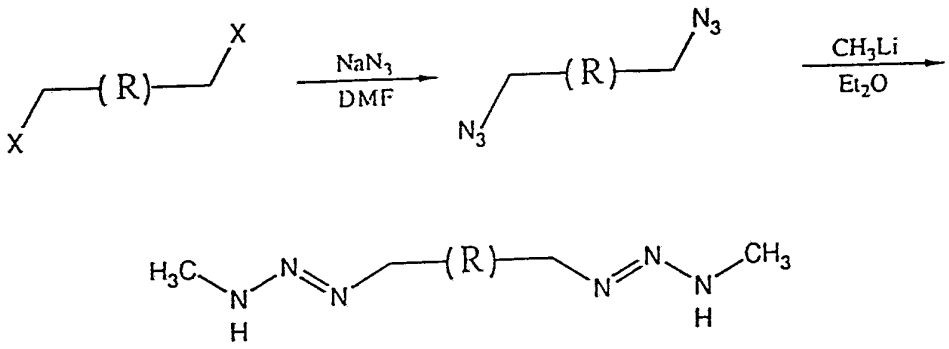
aralkyl or substituted aralkyl (with modifications analogous to those for substituted alkyls), polycyclic aralkyl, aryl groups, and heterocyclic groups of 2-40 non-hydrogen atoms, containing 1-6 rings. Additionally, R may be an acid derivative where the original acid includes, but is not limited to, carboxylic, sulfuric, sulfonic, phosphoric, phosphinic, arsenic, and selenic acids.

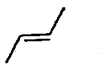
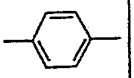
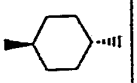
R may also include, in the examples cited above, compounds where R equals R', or R is linked to R' such that a cyclic bistriazene compound is formed. Cases where R equals R' may be expanded to include multivalent metals including, but not limited to, palladium, platinum, titanium, zirconium, silicon, selenium, magnesium, and copper. Several metal species such as cisplatin and titanocene dichloride are clinically active as antineoplastic agents, and the bistriazene moiety may serve as a bidentate ligand for these classes of compounds in order to generate compounds with multiple modes of cytotoxic action. If R is linked to R', polymeric compounds may result in addition to cyclic bistriazenes. The polymers produced would have unusual physical properties due to the hydrolytic instability of triazenes. It can be envisioned that this can be used to prepare polymers which could be implanted, and which would hydrolytically decompose to produce active cytotoxic agent in a time release manner. Similarly, it may be that the polymer would only provide a slowly dissolving matrix. This matrix may be used for structural applications, or to release an entrapped substance.

Furthermore, it should be noted that, while for simplicity, all modifications mentioned above have been discussed as being symmetrical, this need not be the case, and asymmetrical bistriazene molecules are encompassed among the compounds of the present invention.

SYNTHESIS OF BISTRIAZENES

The synthesis of bistriazenes is readily accomplished by the reactions shown below:



R	X	Yield
-	Cl	32%
CH_2	Br	46%
$(CH_2)_2$	Br	58%
$(CH_2)_4$	Br	73%
	Cl	20%
	Cl	50%
	Cl	55%
	OTs	29%

In general, bistriazenes are prepared by the reaction of 1,ω-diazidoalkanes with two equivalents of an alkyllithium. The diazidoalkanes are prepared from the corresponding dihaloalkanes and sodium azide in dimethylformamide solution. For example, the simplest bistriazene, 1,2-bis(methyltriazeno)ethane (BMTE), is prepared by the reaction of 1,2-diazidoethane with two equivalents of methyllithium.

In contrast to simple triazenes, bistriazenes are crystalline solids. X-ray crystal structure determination of BMTE reveals that the molecule adopts a conformation in the solid state which maximizes hydrogen bond interactions with its neighbors. In this regard, BMTE is remarkably similar to polyamines such as spermine, spermidine, and their phosphatidyl derivatives, which are known to bind strongly to DNA.

The synthesis and X-ray crystal structure of bistriazenes are described in Blumenstein et al., Tetrahedron Letters, submitted for publication, and Blumenstein et al., Chemical Communications, submitted for publication, respectively. The synthesis of particular bistriazenes is as follows:

EXAMPLE 1

***Trans*-1,4-bis(methyltriazenomethyl)cyclohexane**

A flask is charged with 3.0 g (6.6 mmole) of *trans*-1,4-di(methyl 4-toluenesulfonate)cyclohexane, 1.08 g (16.6 mmole) of sodium azide, and 50 ml of dimethylformamide (DMF). The mixture is heated at 50°C with stirring under argon for 2 days. The mixture is then diluted with 150 ml of water and extracted four times with 40 ml of petroleum ether. The combined organic layers are dried over Na_2SO_4, filtered, and evaporated to afford a pale yellow oil. The residual oil is dissolved in 100 ml of anhydrous ether and cooled to -20°C under argon. A 1.5 M solution of MeLi in ethyl ether (11 ml, 16.5 mmole) is added to the solution over 0.5 hr. A white precipitate begins to form after a

small amount of the MeLi has been added. The cooling bath is removed and the mixture is allowed to stir overnight. Excess MeLi is quenched by the careful addition of 30 ml of half-saturated NH_4Cl with cooling of the solution. Vigorous gas evolution accompanies the addition of the first several ml of NH_4Cl, and the addition is carried out as quickly as possible. The aqueous layer is then rapidly separated, washed with 40 ml of water, dried over Na_2SO_4, filtered, and evaporated to afford a pale tan solid. The solid is recrystallized from ether/petroleum ether to yield 430 mg (29% yield) of a white solid, mp 72-3°C. Mass spectra (FAB) Calc (M+H) 227.1984, Found 227.2017 ± 0.0023.

EXAMPLE 2

1,4-Bis(methyltriazenomethyl)benzene

A flask is charged with 2.0 g (11.4 mmole) of 1,4-di(chloromethyl)benzene, 1.86 g (28.6 mmole) of sodium azide, and 50 ml of DMF. The mixture is heated at 50°C with stirring under argon overnight. The mixture is worked up and treated with 20 ml of a 1.4 M solution of MeLi (28 mmole) as described above. After workup, a yellow solid is obtained. Crystallization from ether/petroleum ether affords 1.26 g (50% yield) of a pale yellow solid, mp 90-2°C. Mass spectra (FAB) Calc (M+H) 221.1514, Found 221.1558 ± 0.0022

EXAMPLE 3

1,2-Bis(methyltriazenomethyl)benzene

A flask is charged with 7.96 g (45 mmole) of 1,2-di(chloromethyl)benzene, 7.39 g (114 mmole) of sodium azide, and 150 ml of DMF. The mixture is heated at 50°C with stirring under argon overnight. The mixture is worked up, and in 300 ml of anhydrous ether, is treated with 90 ml of a 1.3 M solution of MeLi (117 mmole) as described above. After workup, a yellow-orange oil is obtained. Kugelrohr distillation (110-120°C, 0.5 mm) affords 5.40 g (55% yield) of a pale yellow oil which darkened and became a semi-solid

upon standing. Mass spectra (FAB) Calc (M+H) 221.1514, Found 221.1513 ± 0.0022.

EXAMPLE 4

1,4-Bis(methyltrizeno)butane

A flask is charged with 4.0 g (18.5 mmole) of 1,4-dibromobutane, 3.6 g (55 mmole) of sodium azide, and 50 ml of DMF. The mixture is heated at 50°C with stirring under argon overnight. The mixture is worked up and treated with 45 ml of a 1.3 M solution of MeLi (58 mmole) as described above. After 3 hr the reaction is worked up as described above, and a yellow solid is obtained. Crystallization from ether/petroleum ether affords 1.86 g (58% yield) of a white solid, mp 40-2°C. Mass spectra (FAB) Calc (M+H) 173.1514, Found 173.1510 ± 0.0017.

EXAMPLE 5

1,2-Bis(methyltriazeno)ethane

A flask is charged with 5.0 g (27 mmole) of 1,2-dibromoethane, 3.8 g (58 mmole) of sodium azide, and 50 ml of DMF. The mixture is heated at 50°C with stirring under argon overnight. The mixture is worked up as described above, except that the azide solution is not evaporated down totally. When about 30 ml of solution remains the mixture is treated with 45 ml of a 1.3 M solution of MeLi (58 mmole) as above. After 3 hr the reaction is worked up as described above, and a yellow solid is obtained. Crystallization from ether/petroleum ether affords 1.23 g (32% yield) of an off-white solid, mp 64-6°C. Mass spectra (FAB) Calc (M+H) 145.1201, Found 145.1220 ± 0.0015.

EXAMPLE 6

1,6-Bis(methyltriazeno)hexane

A flask is charged with 10.0 g (41 mmole) of 1,6-dibromohexane, 6.66 g (102 mmole) of sodium azide, and 100 ml of DMF. The mixture is heated at 50°C with stirring under argon overnight. The mixture is worked up and as a

solution in 400 ml of anhydrous ether, is treated with 77 ml of a 1.3 M solution of MeLi (100 mmole) as described above. After 3 hr the reaction is worked up as described above, and a yellow solid is obtained. Crystallization from ether/petroleum ether affords 5.96 g (73% yield) of a white solid, mp 54-5°C.

EXAMPLE 7

1,4-Bis(methyltriazeno)-*trans*-2-butene

A flask is charged with 12.5 g (100 mmole) of 1,4-dichloro-*trans*-2-butene, 14.3 g (220 mmole) of sodium azide, and 200 ml of DMF. The mixture is stirred under argon overnight, worked up, and as a solution in 400 ml of anhydrous ether, is treated with 130 ml of a 1.4 M solution of MeLi (183 mmole) as described above. After 3 hr the reaction is worked up, and a yellow solid is obtained. Crystallization from ether/petroleum ether affords 3.34 g (20% yield) of a pale yellow solid, mp 71-4°C. Mass spectra (FAB) Calc (M+H) 171.1358, Found 171.1397 ± 0.0017.

EXAMPLE 8

1,6-Bis(methyltriazeno)propane

A flask is charged with 10.0 g (49.5 mmole) of 1,3-dibromopropane, 7.08 g (109 mmole) of sodium azide, and 100 ml of DMF. The mixture is stirred under argon overnight, worked up, and as a solution in 400 ml of anhydrous ether, is treated with 80 ml of a 1.4 M solution of MeLi (112 mmole) as described above. After 3 hr the reaction is worked up as described above, and a yellow solid is obtained. Crystallization from ether/petroleum ether affords 3.61 g (46% yield) of a white solid, mp 55-7°C. Mass spectra (FAB) Calc (M+H) 159.1358, Found 159.1360 ± 0.0016.

EXAMPLE 9

1,2-diazidoethane and 1,4-diazidobutane

The procedures described in Examples 4 and 5, above,

were followed.

EXAMPLE 10

1,2-Bis(phenyltriazeno)ethane

A solution of 1.12g (10 mmoles) of 1,2-diazidoethane in 10 ml. of tetrahydrofuran (THF) was cooled to -45°C. To this was added dropwise with stirring 15 ml. of 2.0M phenylmagnesium chloride in THF, and stirring was continued overnight with the temperature being allowed to rise slowly to ambient. The reaction mixture was diluted with 200 ml. of diethylether and the ether solution was washed first with 30 ml. of 10% ammonium chloride in 10% ammonium hydroxide, followed by water (3 x 30 ml.), and then dried over anhydrous sodium sulfate. The solvent was distilled off leaving 2.50g of a pale yellow residue. This was redissolved in 40ml. of ether and allowed to crystallize slowly, which resulted in 1.20 g of powdery crystals, m.p. 125-127°C (dec.). The material migrated as a single spot on TLC and the nmr and the mass spectra were fully consistent with the structure.

EXAMPLE 11

1,4-Bis(phenyltriazeno)butane

A reaction of 1.40 g (10.0 mmoles) of 1,4-diazidobutane with 30 mmoles of phenylmagnesium chloride in 15 ml. of THF was carried out in an identical fashion to the foregoing example of 1,2-bis(phenyltriazeno)ethane. The residue following the evaporation of the ether weighed 2.8 g. This was crystallized from THF/ether mixture and then recrystallized from THF/benzene (2:1) to produce 1.34 g of white crystals, m.p. 116-118°C. (dec.). This material was poorly soluble in diethylether and only soluble in warm (50°C) benzene, and freely in THF. The nmr and mass spectra were fully consistent with the structure.

EXAMPLE 12

1,4-Bis(benzyltriazeno)butane

A solution of 1.40 g (10 mmoles) of 1,4-diazidobutane, in 10 ml. of THF and chilled to -45°C, was treated dropwise with 15 ml of 2.0M benzylmagnesium chloride in THF (30 mmoles). The reaction mixture was magnetically stirred overnight at room temperature. The solvent was removed under vacuum and the residue was dissolved in 200 ml. of diethylether and washed with 30 ml. of ammonium buffer (50 g. ammonium chloride, 178 ml. of 10% ammonium hydroxide and 272 ml. of water), followed by 2 x 30 ml of water. The solution was dried over sodium sulfate and concentrated on a rotary evaporator and dried under high vacuum at room temperature. The crude product was recrystallized from diethylether in the -20° freezer to produce 2.6 g. of white crystals m.p. 71-74°C. The nmr and mass spectra were fully consistent with the structure.

EXAMPLE 13

1,2-Bis(benzyltriazeno)ethane

A solution of 1.12 g (10 mmoles) of 1,2-diazidoethane in 20 ml. of THF was cooled to -60°C. This solution was treated with 15 ml of 2.0 M benzylmagnesium chloride in THF (30mmoles) dropwise with stirring. The reaction mixture was allowed to come to room temperature slowly, and was then cooled again to -60°C, and 10 ml. of ammonium buffer (see preparation above) was added and the reaction was allowed to come to room temperature. The reaction mixture was mixed with 100 ml. of diethylether and the organic layer was separated, dried over anhydrous sodium sulfate, and the solvent was removed on a rotary evaporator. The pink residual oil was subjected to high vacuum overnight, which produced a pale yellow solid. This material was crystallized from ether/pentane (1:1) to produce 1.71 g of colorless powdery solid m.p. 73-75°C. Spectroscopy (nmr and mass) were consistent with the structure.

EXAMPLE 14

1,4-Bis(2-pyridyltriazeno)butane

A solution of 3.95 g (25 mmoles) of 2-bromopyridine in 20 ml of dry pentane was added dropwise to 10 ml. of 2.5M butylithium in hexane at -78°C. To the resulting yellow slurry was added dropwise with stirring 1.75 g (12.5 mmoles) of 1,4-diazidobutane in 10 ml. of pentane. Stirring was continued for 2 hr. at -78° and then the reaction mixture was allowed to warm to room temperature. The reaction mixture was treated with 60 ml. of ammonium buffer which caused a precipitate to appear after the reaction was cooled to -78°C. The solid was filtered off. It was poorly soluble in common solvents such as halomethanes, acetone, methanol and water. It was sparingly soluble in dimethylsulfoxide. The yield was 2.3 g., m.p. 133-136. Spectroscopic data were consistent with the structure.

EXAMPLE 15

1,2-Bis(2-pyridyltriazeno)ethane

A solution of butyllithium in hexane (5 ml., 2.5M) was dissolved in 10 ml. of tetrahydrofuran and cooled to -78°C. To this was added dropwise with stirring a solution of 1.98 g (12.5 mmoles) of 2-bromopyridine in 10 ml. of tetrahydrofuran. The dark yellow solution of the pyridine anion was mixed with a solution of 0.70 g (6.2 mmoles) of 1,2-diazidoethane in 10 ml. of tetrahydrofuran at -78°C. After 1 hr. of stirring the dark green solution was treated with 10 ml. of ammonium buffer. The color changed to yellow. The organic solvent was removed in vacuo, and the precipitate was isolated by filtration and washed with copious quantities of diethylether. The powdery material was finally dried under high vacuum. The yield was 770 mg. This material was not soluble in most solvents, except dimethylsulfoxide. It did not have a sharp melting point since decomposition began before it was reached (rapid

above 100°C). The spectra, however, were fully consistent with the structure.

EXAMPLE 16

1,4-Bis(3-pyridyltriazeno)butane

To 12.5 mmoles of butyllithium in hexane (5 ml. of 2.5 M butyllithium diluted with 10 ml. of hexane) and chilled to -90°C (acetone & liquid nitrogen) was added dropwise 1.978 (12.5 mmoles) of 3-bromopyridine in 10 ml. of hexane. The reaction was further diluted with 20 ml. of hexane to aid stirring at -75°C for 2 hr. and was then treated with 0.876 g. (6.25 mmoles) of 1,4-diazidobutane. The reaction mixture was allowed to stir at room temperature overnight. It was then chilled to -40°C and treated with 10 ml. of the ammonium buffer. This produced two layers and a polymer-like precipitate. The mixture was diluted with 100 ml. of hexane and the layers were separated. The organic phase was washed with water (2 x 20 ml.) and was dried over sodium sulfate. After removal of solvent in vacuo, 0.98 g of oily residue was obtained. This did not contain the desired product. The polymer-like solid, however, was triturated with 30 ml. of diethylether overnight. This resulted in the formation of a pale yellow microcrystalline material, which was filtered and dried under high vacuum. The yield was 0.40 g. of pure bistriazene, which decomposed before melting. The nmr and mass spectra, however, were fully consistent with the structure.

EXAMPLE 17

1,2-Bis(3-pyridyltriazeno)ethane

A solution of 5 ml. of 2.5 M butyllithium in hexane, diluted further with 10 ml. of hexane, was chilled to -90°C. This was treated by dropwise addition with 1.97 g (12.5 mmoles) of 3-bromopyridine in 10 ml. of hexane. The yellow slurry was stirred at -60°C for 1 hr. and then treated by dropwise addition with 0.70 g (6.2 mmoles) of 1,2-diazidoethane. The solution remained yellow, and was

stirred at room temperature overnight. The reaction mixture was then treated with ammonium buffer and the solvents were removed in vacuo. The residue was treated with 50 ml. of diethylether and 10 ml. of water overnight. The residual powder was virtually pure product in a yield of 120 mg (pale orange). The product migrated as a single spot on thin layer chromatography and the nmr and mass spectra were fully consistent with the structure.

EXAMPLE 18

Bis[2-(methyltriazeno)ethyl]ether

A flask was charged with 2.08 g (14 mmole) of bis(2-chloroethyl)ether, 2.73 g (42 mmole) of sodium azide, and 150 ml of dimethylformamide. The mixture was heated at 50°C with stirring under argon for 5 days. The mixture was then diluted with 250 ml of water and extracted four times with 50 ml of pentane. The combined organic layers were dried over sodium sulfate, filtered, and concentrated to afford approximately 25 ml of a solution of the diazide in pentane. The solution was diluted with 100 ml of anhydrous ether and cooled to -20°C under argon. A 3.0 M solution of methyl magnesium bromide in ethyl ether (14 ml, 42 mmole) was added to the solution over 0.5 hr. A white precipitate began to form after a small amount of the reagent had been added. The cooling bath was removed and the mixture was allowed to stir for 2 hr. Excess Grignard reagent was quenched by the careful addition of 50 ml of half-saturated ammonium chloride with cooling of the solution. Vigorous gas evolution accompanied the first several milliliters of ammonium chloride, but the addition was carried out as fast as possible. The aqueous layer was then rapidly separated and washed with 50 ml of pentane. The organic layers were combined, dried over sodium sulfate, filtered and evaporated to afford a pale tan solid. The solid was recrystallized from ether/petroleum ether to yield 1.28 g of a white solid. ^{1}H NMR($CDCl_3$, 200 MHz): 3.195 (br, 4 H), 3.679 (br, 6 H), 7.32 (br, 2 H).

EXAMPLE 19

Bis[2-(methyltriazeno)ethyl]methyl amine

A flask was charged with 2.0 g (10.4 mmole) of bis(2-chloroethyl)methyl amine hydrochloride, 2.03 g (31.2 mmole) of sodium azide, and 100 ml of dimethylformamide. The mixture was heated at 50°C with stirring under argon for 5 days. The mixture was then diluted with 150 ml of 2.5% aqueous sodium hydroxide solution and extracted four times with 50 ml of pentane. The combined organic layers were dried over sodium sulfate, filtered, and concentrated to afford approximately 25 ml of a solution of the diazide in pentane. The solution was diluted with 100 ml of anhydrous ether and cooled to -20°C under argon. A 3.0 M solution of methylmagnesium bromide in ethyl ether (10.4 ml, 31.2 mmole) was added to the solution over 0.5 hr. A white precipitate began to form after a small amount of the reagent had been added. The cooling bath was removed and the mixture was allowed to stir for 2 hr. Excess reagent was quenched by the careful addition of 50 ml of 2.5% NaOH with cooling of the solution. Vigorous gas evolution accompanied the first several milliliters of sodium hydroxide but the addition was carried out as fast as possible. The aqueous layer was then rapidly separated and washed with 50 ml of pentane. The organic layers were combined, dried over sodium sulfate, filtered and evaporated to afford a yellow oil. The oil was distilled under vacuum using a kugelrohr apparatus at 0.1 mm Hg with a pot temperature of 80-120°C to yield 3.120 g of a clear oil. ^{1}H NMR ($CDCl_3$, 200 MHz): 2.67 (br), 2.652 (br), 2.962 (br), 3.406 (br), 3.708 (br), 7.412 (br), 7.716 (br).

BIOLOGICAL ACTIVITY

The bistriazene compounds of the present invention are useful in the treatment of a wide variety of cancers, as shown from the data below.

Clonogenic Assay. The response of a variety of human

tumor cell lines to bistriazenes was determined via the clonogenic assay described in Fiebig et al. (1987) European Journal of Cancer and Clinical Oncology 23: 937-948.

Briefly, the assay system consists of a modified, two-layer soft agar culture system. The bottom layer consists of 1 ml of modified Dulbecco medium supplemented with L-glutamine, containing 10% fetal calf serum and 0.5% agar, in a 35 mm petri-dish. The upper layer contains 2-5 x 10^5 viable human tumor cells suspended in a 1 ml volume, consisting of 0.3% agar, 30% fetal calf serum, and the medium. The drugs to be tested, contained in 1 ml of medium containing 30% fetal calf serum, are included in the upper layer. Control plates are identical, except for the omission of the drugs. The plates are incubated at 37°C in a humidified atmosphere containing 7% carbon dioxide for varying periods (7-21 days). The time in culture is determined by the rate of colony formation in the control plates. At the end of the culture period, the number of colonies in the drug treated cultures is compared to the number of colonies in the control plates, after visualization of the live colonies by staining with tetrazolium chloride.

Three different bistriazenes were examined in the assay. In all cases, the End Group (EG) was methyl, while the Linker was varied:

EG-NH-N=N-Linker-N=N-NH-EG

Linker

p-xyleleno, $-CH_2-C_6H_4-CH_2-$

trans-2-buteno, $-CH_2CH=CHCH_2-$

ethano, $-CH_2CH_2-$

Each of these compounds was evaluated against a panel of human tumor cells, the identity of which is indicated in Figures 1-4. The tumors included those derived from colon cancer, three types of lung cancer, mammary cancer,

ovarian cancer, two types of kidney cancer, a mesothelioma, a gastric cancer, and a sarcoma. These tumors represent some of the most important cancers for which current treatments are inadequate. For comparison, the assays of the various bistriazenes were compared to the response induced in the same tumors by DTIC, a drug employed in clinical practice.

Figure 1 shows dose-response curves obtained in the in vitro clonogenic cytotoxicity assay against several human tumor cell lines employing bis(methyltriazeno)-p-xylene. At a dose of 100 ug/ml, this compound was highly toxic to all tumor cell lines. At a dose of 10 ug/ml, it exhibited toxicity against approximately half of the cell lines examined. Some activity was also evident at a dose of 1 ug/ml in about half the cell lines.

The data in Figure 2 disclose the results obtained with 1,4-bis(methyltriazeno)-*trans*-2-butene. This drug exhibited potent cytotoxic activity against all the tumors tested at 100 ug/ml. This activity persisted at 10 ug/ml, especially for the large cell lung carcinoma LXFL529 and the renal cancer RXF423/17. At a dose of 1 ug/ml, there was still significant activity against the lung cancer. Thus, 1,4- bis(methyltriazeno)-trans-2-butene is a potently active compound, the cytotoxic activity of which is highly specific for certain types of cancers.

Figure 3 discloses the results obtained with bis(methyltriazeno)ethane in the clonogenic assay. This compound was highly cytotoxic at 100 ug/ml to most of the tumor cell lines. Relatively little or no activity was observed, however, in the mesothelioma, the gastric carcinoma, or the renal cancer RXF 423/17. At 10 ug/ml, only marginal, but significant, activity was seen in the large cell lung cancer and in the mammary cancer.

For comparative purposes, the activity of DTIC (5-(3,3-dimethyltriazeno)imidazole-4-carboxamide) was tested in these cell lines. The results are shown in Figure 4. DTIC is used clinically against metastatic melanoma, non-

Hodgkins lymphoma, and soft-tissue sarcomas. At each point, the dose of DTIC was 3 times larger than that of the bistriazenes. Thus, at 300 ug/ml, DTIC was potently cytotoxic on all cell lines. At 30 ug/ml, it showed activity against the gastric carcinoma GXF251/16 and the ovarian cancer OVXF899/9. At 3 ug/ml, it exhibited marginal activity against the gastric cancer. Thus, all of the bistriazenes tested in this assay were at least as potent as DTIC. The bistriazene 1,4-bis(methyltriazeno)-trans-2-butene is highly potent against several tumors, especially the large cell lung carcinoma.

It may be concluded from these data that bistriazenes, as a class of compounds, are cytotoxic agents which exhibit considerable selectivity toward certain tumors. It is also clear from these data that the nature of the Linker is of paramount importance in modulating the activity and selectivity of cytotoxic action of these compounds. The clonogenic assay system facilitates rapid testing of the anti-tumor activities of newly synthesized bistriazenes containing systematically varied EG's and Linkers, in order to establish the chemical and biological characteristics which will result in additional useful drugs.

CHEMICAL ACTIVITY

Crosslinking of Oligonucleotides: The reaction of bistriazenes can afford interstrand crosslinks if the triazene decomposition produces alkydiazonium ions at each end of the Linker chain.

Bistriazenes react with varying efficiency with different oligonucleotides. Unsaturated bistriazenes such as p-xylyl and trans-butenyl produce stable crosslinked species in oligonucleotides. The amount of crosslinked species varies with the oligonucleotide sequence. The level of crosslinking is comparable to that seen with nitrogen mustard, and exceeds that observed with 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea.

The crosslinking of oligonucleotides by bistriazenes

was demonstrated in the following assay system:

A solution of 6.2 ng of ^{32}P-endlabeled oligonucleotide in 0.1 M cacodylic acid buffer (0.1 M NaCl, pH 7.4) was allowed to react with the desired compound dissolved in 1/10 volume DMSO. Final concentrations of the compounds in the oligonucleotide solution were 0.1 mM NMUST, 1.0 mM CCNU, or 10 mM bistriazene. Reactions were incubated at 37°C for 42 hours, and analyzed by denaturing polyacrylamide gel electrophoresis (20% gel) followed by autoradiography. the gels were intentionally overexposed to better visualize bands corresponding to interstrand crosslinks. Large amounts of unreacted oligonucleotides were also visualized under these conditions.

As shown in Figure 5, the results obtained with nitrogen mustard and the p-xylyl bistriazene derivative demonstrate that the oligonucleotides were stably covalently crosslinked by both the nitrogen mustard, a known crosslinking agent, as well as by the p-xylyl bistriazene of the instant invention. As can also be seen from Figure 5, CCNU, a clinically employed DNA interstrand crosslinking agent, was not as effective at forming crosslinks as the p-xylyl bistriazene derivative.

All compounds examined caused extensive DNA strand breakage, because of which labile adducts were not observable.

Plasmid DNA Strandbreaking. DNA strand breaks may occur via the hydrolysis of labile alkylation sites. A single strand break allows the relaxation of supercoiled DNA to afford a nicked open circular form. Double strand breakage producing linear plasmid DNA occurs upon the hydrolysis of two labile alkylation sites close to one another on opposite DNA strands. These alkylation events may be either an interstrand crosslink, or discrete, but closely located, monoalkylations.

Dialkyltriazenes afford more strand breakage than alkylsulfates and sulfonates. Bistriazenes are

approximately 10-200 times more efficacious at producing strand breaks than dialkyltriazenes. Bistriazenes afford significant quantities of linear DNA, whereas simple dialkyltriazenes produce only small amounts of the linear form, and only traces are detectable in the reaction of alkylsulfates with plasmid DNA. Restriction endonuclease treatment of bistriazene-modified DNA suggests that linearization is not highly specific for sequences on the plasmid.

The supercoiled plasmid strand break assay was carried out in a solution of 0.15 ug of pBR322 DNA in 9.5 ul of TE buffer (10 mM) Tris, 0.1, mM EDTA, pH 7.4) prepared at room temperature. A 0.5 ul aliquot of compound in DMSO was added, the solution vortexed lightly, and the samples incubated at 37° for 48 hours. Loading buffer (2 ul, 40% glycerol, and 1% bromphenol blue in TAE buffer) was added to each sample, and a 3 ul aliquot was analyzed by agarose gel electrophoresis (0.9% gel, 1.5 ug ethidium bromide/ml gel), and visualized by fluorescence.

The experimental results shown in Figure 6 indicate that the bistriazenes examined afford higher levels of DNA modification than do simple dialkyltriazenes such as dimethyltriazene, and that the bistriazenes afford far more linearized DNA, indicated labile alkylation events on opposite strands of the DNA in close proximity to one another. These alkylation events may be a labile interstrand crosslink or discrete alkylation events near one another on opposite strands.

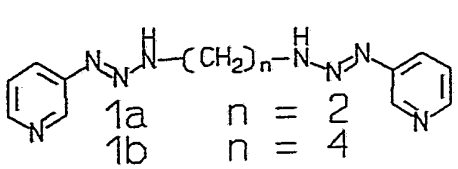
This suggests interaction of the bistriazine with DNA prior to forming active alkylating agent rather than simple hydrolysis to alkyldiazonium ion.

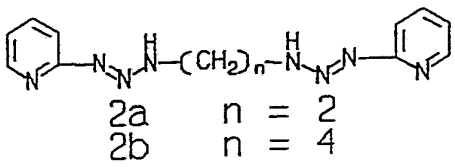
BISTRIAZENES POSSESSING ENHANCED CHEMICAL STABILITY AND THE SAME OR GREATER CYTOTOXIC EFFECTS

The bistriazenes described above are highly chemically

reactive. It was therefore deemed desirable to prepare bistriazenes possessing the same or greater cytotoxic effects, but which are more stable chemically. The rationale for the preparation of such bistriazenes is described below.

The bistriazenes described supra have $-CH_3$ (methyl) as the EG. Since the EGs appeared to have little influence on the reactivity of these molecules toward DNA, it was reasoned that they could be employed to modulate the reactivity of bistriazenes. It was further reasoned that electron attracting groups would increase the chemical stability of the bistriazenes. Accordingly, bistriazenes, where the linker was ethano [$(CH_2)_2$] or butano [$(CH_2)_4$], and the EG groups were phenyl (C_6H_5) or benzyl ($C_6H_5CH_2$), were prepared. These compounds proved to be much more stable toward decomposition than those where the EG was methyl. It was further reasoned that the stability of bistriazenes could be enhanced even more if the EG could be protonated at physiological pH. The reason for this hypothesis is that if the EG had a positive charge, it would be much more difficult to decompose the bistriazene molecule since this reaction requires the addition of a proton to the triazene moiety. The bistriazenes bis(3-pyridyltriazeno)ethane *(1a)*, bis(3-pyridyltriazeno)butane *(1b)*, and the isomeric bis(2-pyridyltriazeno)ethane *(2a)* and bis(2-pyridyltriazeno)butane *(2b)* were prepared by the reaction of the corresponding pyridyl lithium with 1,2-diazidoethane or 1,4-diazidobutane via procedures described above.





The rates of decomposition of the bistriazenes (*1*) and (*2*) were measured in buffer at pH 7.4. Bistriazenes *1* had half-lives of approximately 5 hours under those conditions,

while bistriazenes *2* were somewhat less stable, having half-lives of about 5 minutes. This is compared to bistriazenes where EG is methyl, which have half-lives on the order of several seconds under these conditions. The nature of the linker had little effect on the decomposition rate.

The enhanced chemical stability of bistriazenes (*1*) and (*2*) had little effect on their ability to interact with DNA. This was determined by the reaction of the bistriazenes with the supercoiled plasmid pBR322. For example, the ability of bistriazene (*1a*) to open, linearize, and finally to shear the plasmid (Figure 7) was essentially identical to that observed for 1,2-bis(methyltriazeno)ethane (Figure 6). Thus, electron withdrawing groups EG stabilize bistriazenes with respect to proteolytic decomposition in buffer, while leaving unaffected the DNA-damaging activity.

Furthermore, preliminary cytotoxicity data obtained via the clonogenic assay described above revealed that bistriazenes (*1a*) and (*1b*) are potently cytotoxic. These data are shown in Table 1, below.

Table 1

Cytotoxicity of bis(3-pyridyltriazeno)ethane(*1a*) and bis(3-pyridyltriazeno)butane(*1b*) on Human Tumor Cell Lines

Drug	Cell Line[a]	Test/Control (%) at Drug Concentration (μg/ml) 1.0	10.0	100.0
1a	LXFL 529	51	0+++	0+++
	PRCL DU145Y	84	44+	0+++
	RXF 1220	99	74	4+++
1b	LXFL 529	71	1+++	1+++
	PRCL DU145Y	94	62	4+++
	RXF 1220	82	70	8+++
DTIC[b]	LXFL 529	63	35+	8+++
	PRCL DU145X	70	44+	43+
	RXF 1220	54	44+	24+
CYCM[c]	LXFL 529	115	94	19++
	PRCL DU145X	108	90	47+
	RXF 1220	67	51	9+++

[a] LXFL 529 is a large cell lung cancer line, PRCL DU145X is a prostate carcinoma, and RXF 1220 is a renal carcinoma.

[b] DTIC (5-(dimethyltriazeno) imidazole-4-carboxamide) is a clinically used drug, and is positive in this panel.

[c] CYCM, 4-hydroperoxycyclophosphamide, is an activated form of the well-known cytotoxic agent cytoxan.

Thus, electron attracting EGs in the bistriazenes encompassed by the present invention impart desirable enhanced chemical stability without affecting the DNA damaging effect of these drugs.

Specifically, the following types of EGs fulfill this requirement:

(a) Phenyl and substituted phenyl groups:

The substituents can include electron attracting moieties such as one or more nitro ($-NO_2$) groups, one or more halogen atoms (such as fluorine, chlorine, bromine, or iodine), one or more cyano (-CN) groups, one or more trifluoromethyl groups, one or more carboxyl groups or esters or amides derived therefrom, or various combinations of these substituents.

(b) Arylalkyl or substituted arylalkyl groups:

These include benzyl ($C_6H_5CH_2$) and benzyls substituted as described in (a) above. Also included are condensed ring arylalkyls such as naphthylmethyl.

(c) Heterocyclic ring systems:

These include, but are not limited to, 2-pyridyl, 3-pyridyl and 4-pyridyl, 4-imidazolyl and 4-imidazolyl-5-carboxamide, various EGs derived from pyrimidines (cytosine, thymidine and uracil) and purines (adenine and guanine), and various oligonucleotides derived from combinations of purines and pyrimidines. The oligonucleotides can be held together by normal phosphate links, or by methylphosphonate or phosphorothioate links.

(d) Amine and polyamine-derived EGs:

Since amines can be protonated at physiological pH, and would thus fulfill the same stabilizing role, groups such as 2-aminopropyl, and 2-(N,N-dialkylamino)propyl such as 2-(N,N-dimethylamino)propyl or 2-(N,N-diethylamino)propyl are useful. Also contemplated are groups which have more than one amino group, such as 2-(N-[4-(N'-propylamino)butyl]amino)propyl($-CH_3CH_2CH_2NH-CH_2CH_2CH_2CH_2NHCH_2CH_2CH_2-$). These are analogs to natural polyamines.

As a means of enhancing the reactivity of bistriazenes toward DNA, resulting in drugs that would require administration in lower doses to achieve similar therapeutic results, the present inventors have modified the linker in the bistriazene structure. Specifically, bistriazene (*3*) was prepared:

$$CH_3-N=N-NH-CH_2CH_2N(CH_3)-CH_2CH_2NH-N=N-CH_3 \qquad (3)$$

This compound is at least 100 times more reactive toward pBR322 than 1,2-bis(methyltriazeno)ethane (cf. Figures 6 and 8). At the beginning of this application there is discussed a paper by Vaughan et al. (1984) *J. Med. Chem.* 27:357-63 which describes a bistriazene in which the linker is the same as in bistriazene (*3*). The Vaughan et al bistriazene is, however, substantially different from (*3*) for the following reasons:

1. The saturated nitrogens therein have additional substituents (methyl groups). Such bistriazenes would require a double metabolic demethylation in order to become alkylating agents--a very unlikely scenario. In contrast, the bistriazenes of the present invention require H atoms instead of alkyl groups in that position.

2. The synthetic method is very different, as is the chemistry of the Vaughan et al bistriazene, as compared to the bistriazenes of the present invention.

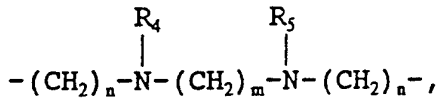
3. The Vaughan et al. bistriazene would be expected to act like a monotriazene in biological systems, i.e., it would be a simple alkylating agent.

Thus, the bistriazenes of the present invention include those with the following linker modifications:

(a) The linker shown in structure (3), together with various modifications of that scheme, i.e., a central nitrogen flanked by $(CH_2)_n$, where n=1-5.

(b) Instead of a methyl on the central nitrogen, the latter can be substituted by hydrogen or other normal alkyl groups from methyl to pentyl.

(c) The linker can also possess more than one nitrogen. Specifically, the following linker, which would closely mimic a polyamine structure, is contemplated:



where n=1-5, m=1-5, and R_4 and R_5=normal alkyl up to pentyl.

(d) Instead of the central atom being N, it can also be oxygen, sulfur or selenium. In those cases, no other substituents on the oxygen atom would be possible, except those forming the linker. In the case of sulfur, however, the atom could be oxidized to the sulfoxide or the sulfone.

PHARMACEUTICAL PREPARATIONS

The bistriazene compounds of the present invention, or physiologically acceptable salts thereof, can be formulated into a pharmaceutical composition comprising an effective anti-cancer amount of the compound and a pharmaceutically acceptable carrier. An effective anti-cancer amount of the pharmaceutical composition will be administered to the subject, human, animal, or mammal, in a manner which inhibits cancer cell growth or replication. The amount of the compound and the specific pharmaceutically acceptable carrier will vary depending upon the host and its condition, the mode of administration, and the type of cancer being treated.

In a particular aspect, the pharmaceutical composition comprises a bistriazene anti-cancer compound or physiologically acceptable salt thereof in effective unit dosage form. As used herein, the term "effective unit dosage" or "effective unit dose" is denoted to mean a predetermined anti-cancer amount sufficient to be effective against the cancer in vivo. Pharmaceutically acceptable carriers are materials useful for the purpose of administering the medicament, which are preferably non-toxic, and may be liquid materials which are otherwise inert and medically acceptable, and are compatible with the active ingredients.

The pharmaceutical compositions of the present invention can also contain an anti-cancer effective amount of at least one conventional alkylating agent, such as chlorambucil, melphalan, uracil, mustard NF, cyclophosphamide, mechlorethamine hydrochloride, carmustine (BCNU), lomustine, dacarbazine (DTIC), thiotepa NF, and busulfan, or combinations thereof. Pharmaceutical compositions of the present invention can also contain, in addition to a bistriazene compound or physiologically acceptable salt thereof, at least one conventional chemotherapeutic agent other than an alkylating agent, as would be apparent to one of ordinary skill in the art of cancer chemotherapy. Also contemplated in the present invention are pharmaceutical compositions containing a bistriazene compound or physiologically acceptable salt thereof, at least one conventional alkylating agent, and at least one conventional chemotherapeutic agent other than an alkylating agent. Pharmaceutical compositions of the present invention can also include those wherein more than one of the bistriazene cmpounds described supra are employed in conjunction with one another, either alone or in combination with at least one conventional alkylating agent and/or at least one conventional chemotherapeutic agent other than an alkylating agent. All pharmaceutical compositions of the present invention can also contain

other active ingredients such as antimicrobial agents and other agents such as preservatives, and can be employed in treating cancer in a mammal, including humans.

These pharmaceutical compositions may take the form of a solution, an emulsion, suspension, ointment or cream. They may be administered parenterally, orally or topically, as an aerosol, spray, or drops, said parenteral administration being conducted intraperitoneally, intramuscularly, subcutaneously, intravenously, intraarticularly, intraarterially, or transdermally, depending upon whether the preparation is used to treat internal or external cancers.

The compositions may contain the compound in an amount of from about 0.1 % - about 99% by weight of the total composition, preferably about 1 to about 90% by weight of the total composition. For parenteral injection, the bistriazene compound can be dissolved in a pharmaceutically suitable carrier such as purified corn oil, propylene glycol, triolene, or dimethyl sulfoxide, and the dose may be about 0.1 mg to about 1000 mg per kilogram per day. If administered intraperitoneally, the compounds may be dissolved in a suitable vehicle, as above, and the dose may be about 1 mg to about 500 mg per kilogram per day. If injected intramuscularly, the compounds can be dissolved in oil or another compatible vehicle, and the dose can be about 0.1 mg to about 1000 mg per kilogram per day. In any case, injections can be carried out once or several times per day over a five day course depending upon the route of administration and the condition of the patient. After such courses, a recovery period of various length may be necessary. Additional courses may then be required under specific conditions. Total adult doses can range from about 0.1 to about 5000 mg, with dosages in the range of from about 10 to about 1000 mg being preferred. For certain particular applications, oral administration of bistriazenes encapsulated in liposomes or time-release formulations or dispersed in compatible emulsions together

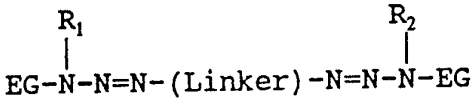
with stabilizing and/or dispersing agents may be the method of choice.

For topical application, to treat surface lesions such as basal cell and squamous cell carcinomas or non-metastasized melanomas, as well as certain non-malignant conditions which are characterized by rapid cell proliferation but which may not be amenable to surgical treatment, bistriazenes may be formulated in oil or cream.

The invention being thus described, it will be obvious that the same may be varied in many ways. Such variations are not to be regarded as a departure from the spirit and scope of the invention, and all such modifications as would be obvious to one skilled in the art are intended to be included within the scope of the following claims.

WHAT IS CLAIMED:

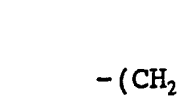
1. A bistriazene compound or a physiologically acceptable salt thereof of the formula:



wherein

said Linker is selected from the group consisting of

, where n=1-5, and R_3 is selected from the group consisting of hydrogen and a C_1-C_5 n-alkyl,

 , where n=1-5, m=1-5, and R_4 and R_5 each = C_1-C_5 n-alkyl,

$-(CH_2)_n-O-(CH_2)_n-$, where n=1-5,

$-(CH_2)_n-S-(CH_2)_n-$, where n=1-5,

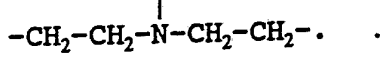
$-(CH_2)_n-Se-(CH_2)_n-$, where n=1-5,

, where n=1-5, and

$-(CH_2)_n-SO_2-(CH_2)_n-$, where n=1-5; and

said EG is identical or independently selected from the group consisting of a phenyl group, a substituted

phenyl group, an arylalkyl group, a substituted arylalkyl group, a condensed ring arylalkyl group, a heterocyclic group, an amine group, and a polyamine.

2. The compound or physiologically acceptable salt of claim 1, wherein said Linker is  .

3. The compound or physiologically acceptable salt of claim 1, wherein the substituents of said substituted phenyl group and said substituted arylalkyl group are electron attracting moieties.

4. The compound or physiologically acceptable salt of claim 3, wherein said electron attracting moieties are selected from the group consisting of one or more nitro groups, one or more halogen atoms, one or more cyano groups, one or more trifluoromethyl groups, one or more carboxyl groups, esters, or amides, and combinations thereof.

5. The compound or physiologically acceptable salt of claim 4, wherein said halogen atoms are selected from the group consisting of fluorine, chlorine, bromine, and iodine.

6. The compound or physiologically acceptable salt of claim 1, wherein said arylalkyl group is a benzyl group.

7. The compound or physiologically acceptable salt of claim 1, wherein said substituted arylalkyl group is a substituted benzyl group.

8. The compound or physiologically acceptable salt of claim 1, wherein said condensed ring arylalkyl group is a naphthylmethyl group.

9. The compound or physiologically acceptable salt of claim 1, wherein said heterocyclic group is selected from the group consisting of a 2-pyridyl group, a 3-pyridyl group, a 4-pyridyl group, a 4-imidazolyl group, a 4-imidazolyl-5-carboxamide group, a pyrimidine, a purine, and an oligonucleotide.

10. The compound or physiologically acceptable salt of claim 9, wherein said oligonucleotide comprises purines and pyrimidines.

11. The compound or physiologically acceptable salt of claim 9, wherein said oligonucleotide is held together by phosphate linkages, methylphosphonate linkages, or phosphorothioate linkages.

12. The compound or physiologically acceptable salt of claim 1, wherein said amine is selected from the group consisting of a 2-aminopropyl group and a 2-(N,N-dialkylamino)propyl group.

13. The compound or physiologically acceptable salt of claim 12, wherein said 2-(N,N-dialkylamino)propyl group is selected from the group consisting of a 2-(N,N-dimethylamino)propyl group and a 2-(N,N-diethylamino)propyl group.

14. The compound or physiologically acceptable salt of claim 1, wherein said polyamine is an analog of a natural polyamine.

15. The compound or physiologically acceptable salt of claim 14, wherein said polyamine is 2-(N-[4-(N'-propylamino)butyl]amino)propyl (-$CH_3CH_2CH_2NHCH_2CH_2CH_2CH_2NH$-$CH_2CH_2CH_2$-).

16. The compound or physiologically acceptable salt of claim 1, wherein said compound or physiologically acceptable salt is selected from the group consisting of

1,2-diazidoethane,
1,4-diazidobutane,
1,2-Bis(phenyltriazeno)ethane,
1,4-Bis(phenyltriazeno)butane,
1,4-Bis(benzyltriazeno)butane,
1,2-Bis(benzyltriazeno)ethane,
1,4-Bis(2-pyridyltriazeno)butane,
1,2-Bis(2-pyridyltriazeno)ethane,
1,4-Bis(3-pyridyltriazeno)butane,
1,2-Bis(3-pyridyltriazeno)ethane,
Bis[2-(methyltriazeno)ethyl]ether, and
Bis[2-(methyltriazeno)ethyl]methyl amine.

17. A pharmaceutical composition, comprising an anti-cancer effective amount of at least one bistriazene compound of claim 1, and a pharmaceutically acceptable carrier.

18. The pharmaceutical composition of claim 17, wherein said bistriazene compound is selected from the group consisting of

1,2-diazidoethane,
1,4-diazidobutane,
1,2-Bis(phenyltriazeno)ethane,
1,4-Bis(phenyltriazeno)butane,
1,4-Bis(benzyltriazeno)butane,
1,2-Bis(benzyltriazeno)ethane,
1,4-Bis(2-pyridyltriazeno)butane,

1,2-Bis(2-pyridyltriazeno)ethane,
1,4-Bis(3-pyridyltriazeno)butane,
1,2-Bis(3-pyridyltriazeno)ethane,
Bis[2-(methyltriazeno)ethyl]ether, and
Bis[2-(methyltriazeno)ethyl]methyl amine.

19. The pharmaceutical composition of claim 17, comprising an anti-cancer effective amount of said bistriazene compound, and at least one of an anti-cancer effective amount of an alkylating agent selected from the group consisting of bis(2-chloroethyl)nitrosourea, mitomycin, cyclophosphamide, ifosphamide, and any other conventional chemotherapeutic alkylating agent, and a conventional chemotherapeutic agent other than an alkylating agent.

20. The pharmaceutical composition of claim 19, wherein said bistriazene compound is selected from the group consisting of

1,2-diazidoethane,
1,4-diazidobutane,
1,2-Bis(phenyltriazeno)ethane,
1,4-Bis(phenyltriazeno)butane,
1,4-Bis(benzyltriazeno)butane,
1,2-Bis(benzyltriazeno)ethane,
1,4-Bis(2-pyridyltriazeno)butane,
1,2-Bis(2-pyridyltriazeno)ethane,
1,4-Bis(3-pyridyltriazeno)butane,
1,2-Bis(3-pyridyltriazeno)ethane,
Bis[2-(methyltriazeno)ethyl]ether, and
Bis[2-(methyltriazeno)ethyl]methyl amine.

21. A method for treating cancer in a mammal, comprising administering to said mammal an anti-cancer effective amount of at least one bistriazene compound or physiologically acceptable salt thereof according to claim 1.

22. The method of claim 21, wherein said bistriazene compound is selected from the group consisting of

1,2-diazidoethane,
1,4-diazidobutane,
1,2-Bis(phenyltriazeno)ethane,
1,4-Bis(phenyltriazeno)butane,
1,4-Bis(benzyltriazeno)butane,
1,2-Bis(benzyltriazeno)ethane,
1,4-Bis(2-pyridyltriazeno)butane,
1,2-Bis(2-pyridyltriazeno)ethane,
1,4-Bis(3-pyridyltriazeno)butane,
1,2-Bis(3-pyridyltriazeno)ethane,
Bis[2-(methyltriazeno)ethyl]ether, and
Bis[2-(methyltriazeno)ethyl]methyl amine.

23. The method of claim 21, wherein said bistriazene compound is administered in the form of a solution, emulsion, suspension, ointment, or cream.

24. The method of claim 21, wherein said bistriazene compound is administered parenterally, orally, or topically, as an aerosol, spray, or drops, said parenteral administration being conducted intraperitoneally, intramuscularly, subcutaneously, intravenously, intraarterially, intraarticularly or transdermally.

25. The method of claim 24, wherein said bistriazene compound is administered parenterally, dissolved in oil or other pharmaceutically acceptable carrier in a dose of from about 0.1 mg to about 1000 mg per kilogram per day.

26. The method of claim 24, wherein said bistriazene compound is administered intraperitoneally, dissolved in a pharmaceutically acceptable carrier, in a dose of from about 1 mg to about 500 mg per kilogram per day.

27. The method of claim 24, wherein said bistriazene compound is administered intramuscularly, dissolved in oil or another compatible vehicle, in a dose of from about 0.1 mg to about 1000 mg per kilogram per day.

28. The method of claim 24, wherein said bistriazene compound is administered orally, encapsulated in liposomes or time-release formulations, or dispersed in compatible emulsions together with stabilizing and/or dispersing agents.

29. The method of claim 24, wherein said bistriazene compound is administered topically, formulated in an oil or cream.

30. The method of claim 25, 26, 27, 28, or 29, wherein said bistriazene compound is administered once or several times per day over a five day course.

31. A method of inhibiting breakage or digestion of DNA or proteins, comprising treating said DNA or proteins with a bistriazene compound of claim 1.

32. A method of producing chemical polymers, comprising treating their monomeric constituents with a bistriazene compound of claim 1.

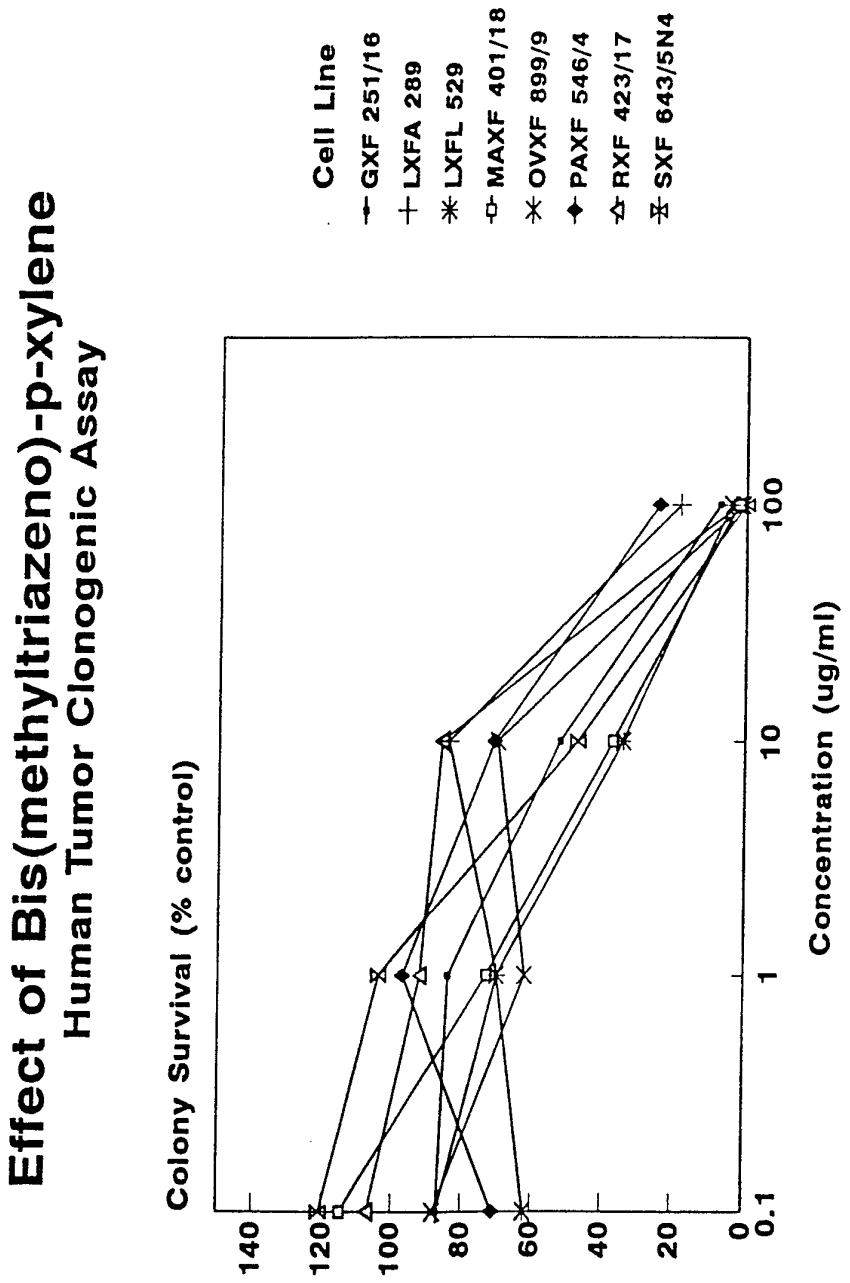

Effect of Bis(methyltriazeno)-p-xylene
Human Tumor Clonogenic Assay
Cell Line
GXF 251/16
LXFA 289
LXFL 529
MAXF 401/18
OVXF 899/9
PAXF 546/4
RXF 423/17
SXF 643/5N4
Colony Survival (% control)
140
120
100
80
60
40
20
0
0.1
1
10
100
Concentration (ug/ml)

FIGURE 1

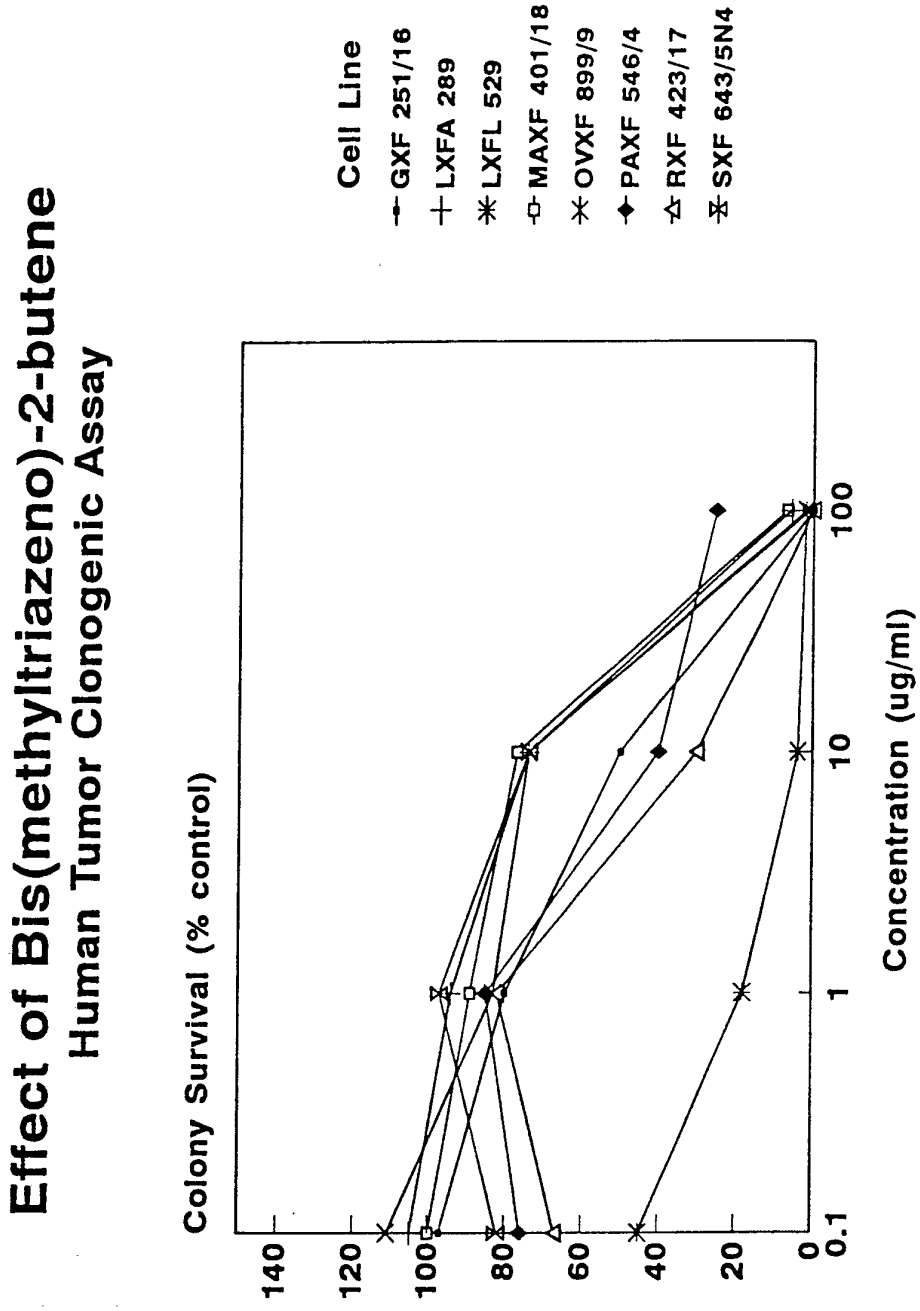

Effect of Bis(methyltriazeno)-2-butene
Human Tumor Clonogenic Assay
Cell Line
GXF 251/16
LXFA 289
LXFL 529
MAXF 401/18
OVXF 899/9
PAXF 546/4
RXF 423/17
SXF 643/5N4
Colony Survival (% control)
140
120
100
80
60
40
20
0
0.1
1
10
100
Concentration (ug/ml)

FIGURE 2

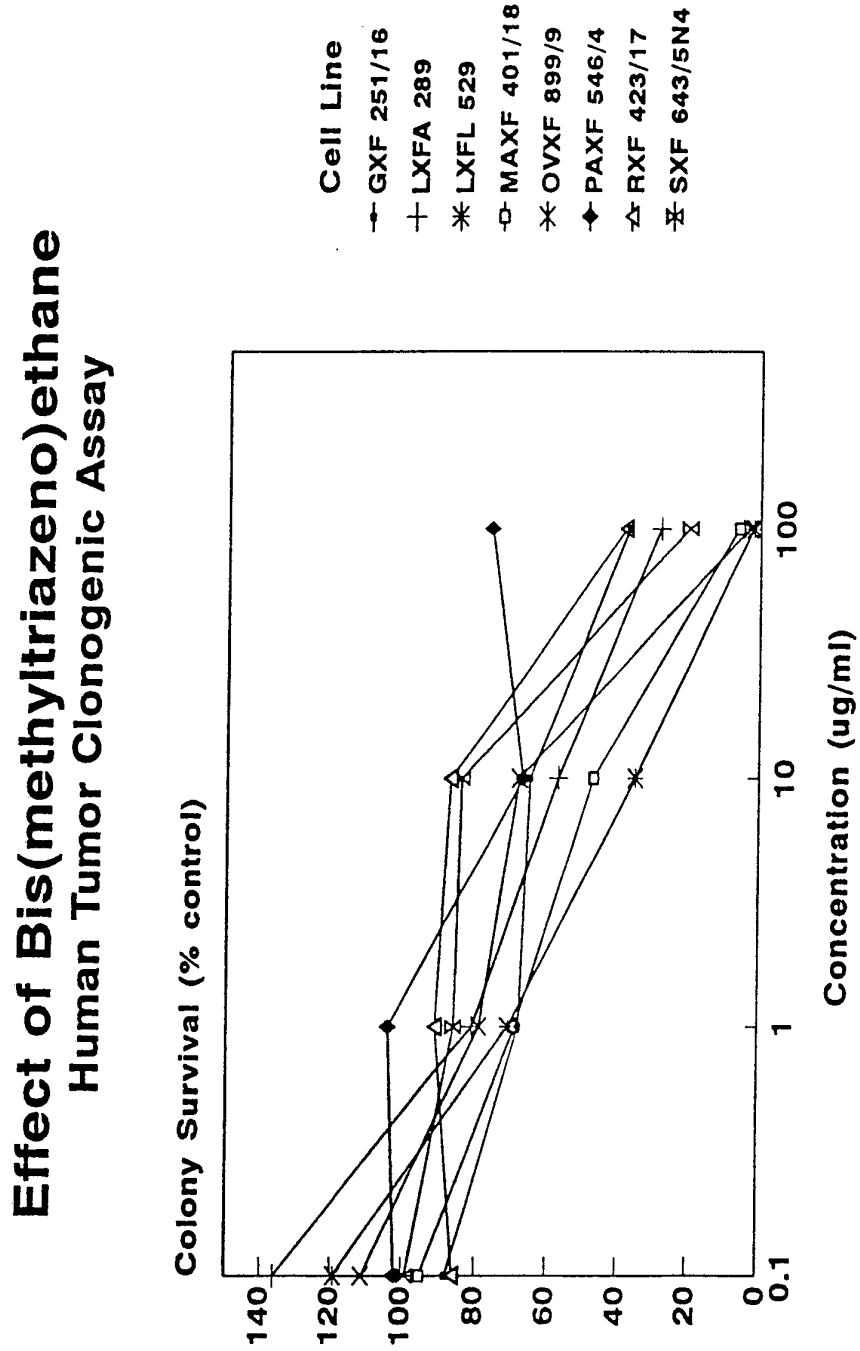

Effect of Bis(methyltriazeno)ethane
Human Tumor Clonogenic Assay
Cell Line
GXF 251/16
LXFA 289
LXFL 529
MAXF 401/18
OVXF 899/9
PAXF 546/4
RXF 423/17
SXF 643/5N4
Colony Survival (% control)
140
120
100
80
60
40
20
0
0.1
1
10
100
Concentration (ug/ml)

FIGURE 3

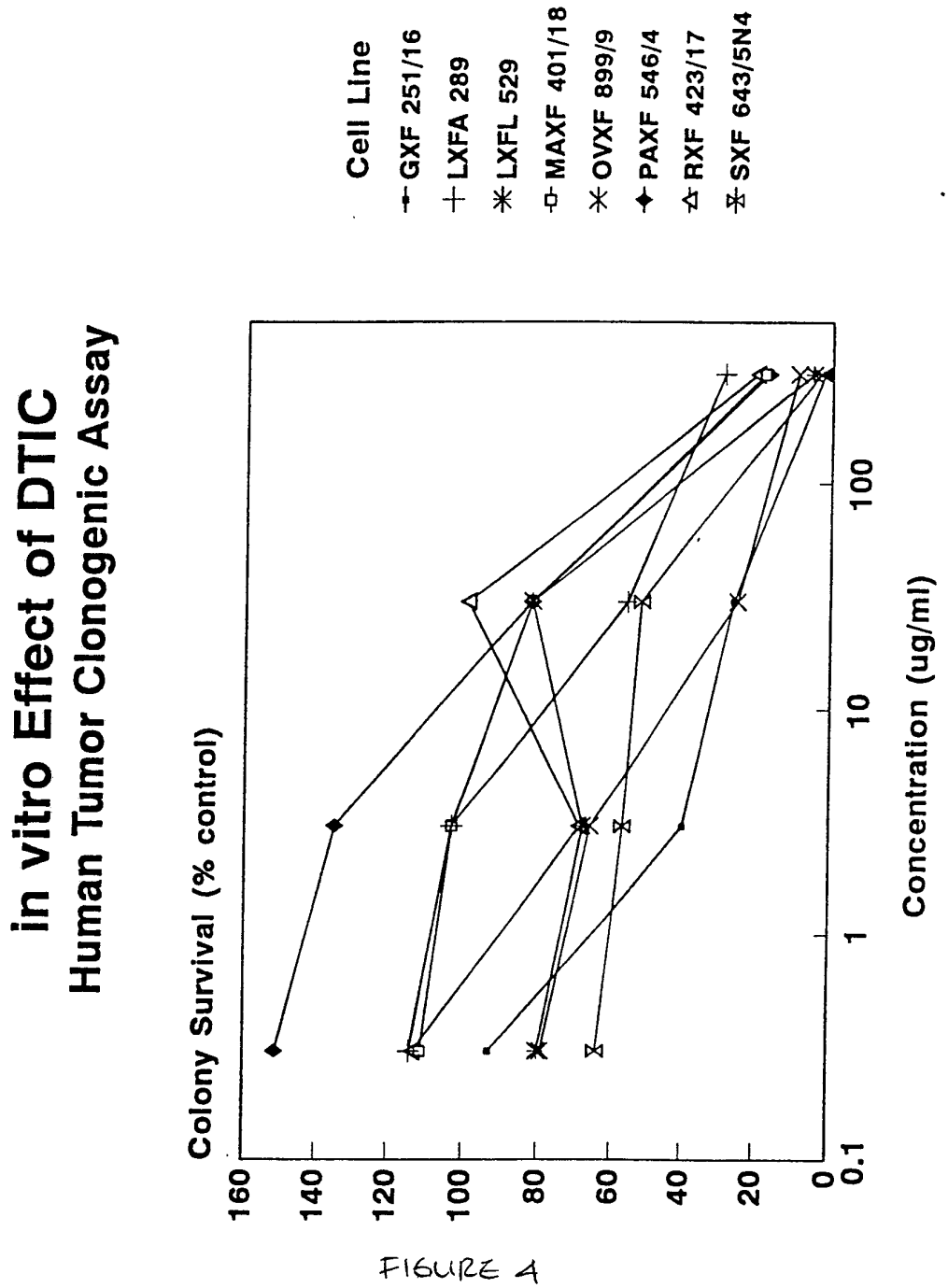
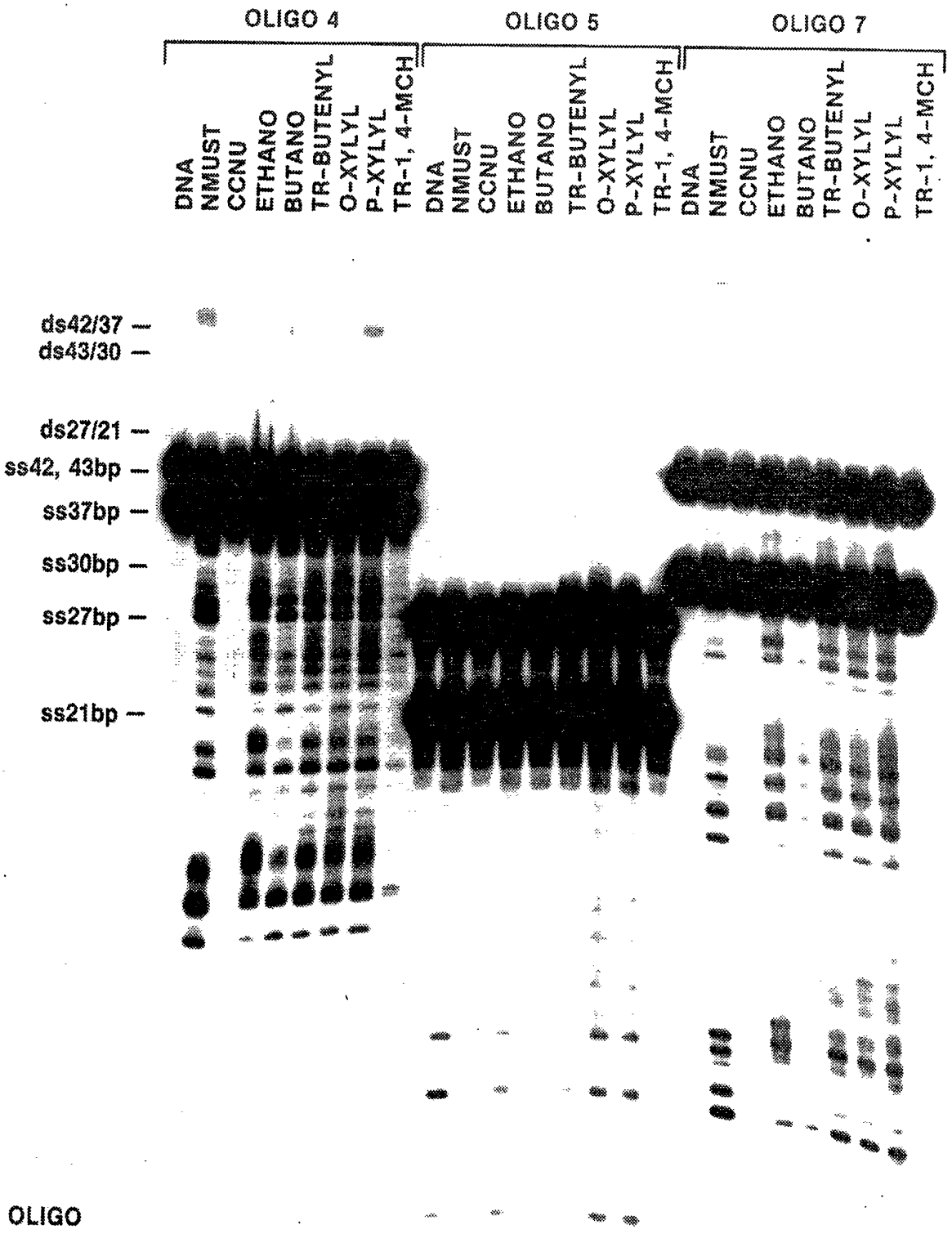

In vitro Effect of DTIC
Human Tumor Clonogenic Assay
Cell Line
GXF 251/16
LXFA 289
LXFL 529
MAXF 401/18
OVXF 899/9
PAXF 546/4
RXF 423/17
SXF 643/5N4
Colony Survival (% control)
160
140
120
100
80
60
40
20
0
0.1
1
10
100
Concentration (ug/ml)

FIGURE 4



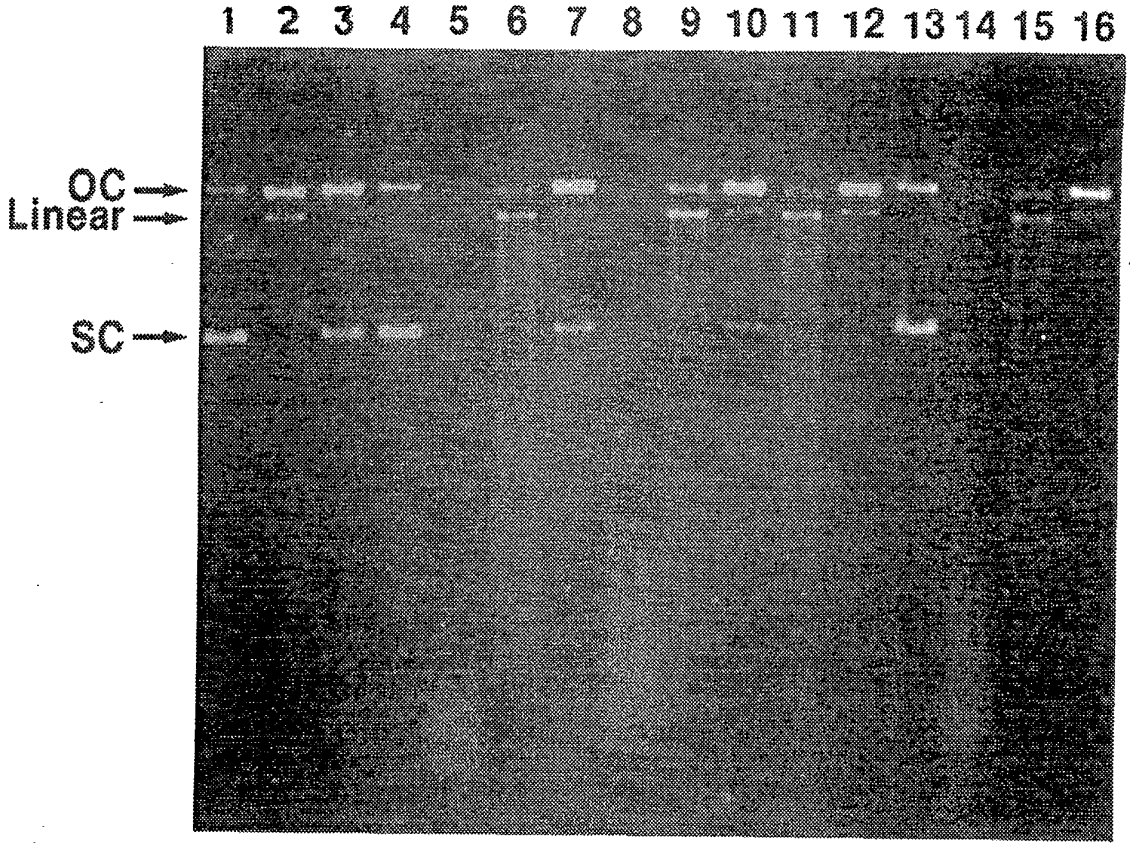
OLIGO

4 5' CTTTAATTTTACTGGTACAGTTTCAATAGGACTAATGGGAAC 3' 42 BP
3' ATGACCATGTCAAAGTTATCCTGATTACCCTTGGTAC 5' 37 BP

5 5' CGATCATATGGATATCATGCATGAGCT 3' 27 BP
3' TAGTATACCTATAGTACGTAC 5' 21 BP

7 5' TCGACTATAGTATTTTCCTGATTCCAGCACTGACTAATTTATC 3' 43 BP
3' GATATCATAAAAGGACTAAGGTCGTGACTG 5' 30 BP

FIGURE 5



LANE	COMPOUND	CONCENTRATION IN mM
1	CONTROL	-
2	DIMETHYLTRIAZENE	5.0
3	"	0.5
4	"	0.05
5	1,2-BIS(METHYLTRIAZENO)ETHANE	5.0
6	"	0.5
7	"	0.05
8	1,4-BIS(METHYLTRIAZENO)-TRANS-2-BUTENE	5.0
9	"	0.5
10	"	0.05
11	1,6-BIS(METHYLTRIAZENO)HEXANE	5.0
12	"	0.5
13	"	0.05
14	α,α'-BIS(METHYLTRIAZENO)-P-XYLENE	5.0
15	"	0.5
16	"	0.05

FIGURE 6

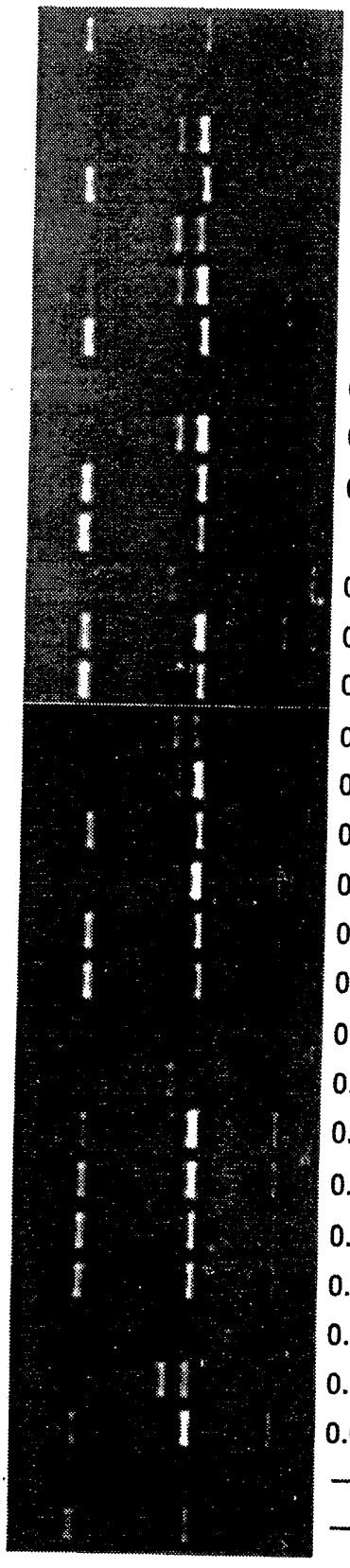
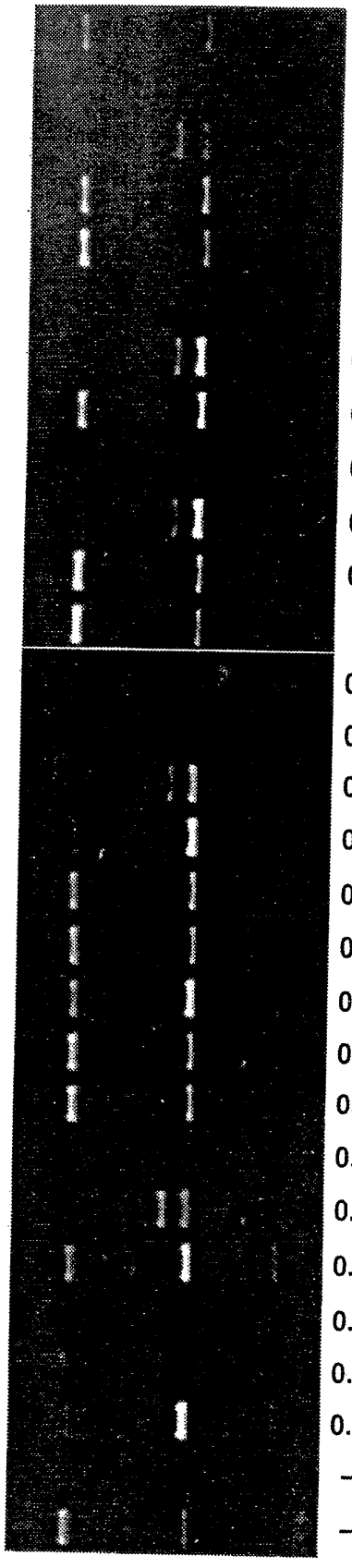


FIGURE 7

SUBSTITUTE SHEET



Concentration (in mM)	Compound
—	Control
5.10^{-3}	Bis[2-(methyltriazeno)ethyl]-methylamine
5.10^{-4}	
5.10^{-5}	
5.10^{-6}	
0.5	Bis[2-(methyltriazeno)ethyl]-ether
0.05	
0.005	
0.5	1-Methyl-3-(2-dimethylamino)-ethyltriazene
0.05	
0.005	
—	Control
0.5	α,α'-Bis(allyltriazeno)-p-xylene
0.05	
0.005	
0.5	1-Allyl-3-*n*-butyltriazene
0.05	
0.005	
0.5	1,3-Dimethyltriazene
0.05	
0.005	
0.5	1,2-Bis(methyltriazeno)ethane
0.05	
0.005	
0.5	α,α'-Bis(methyltriazeno)-p-xylene
0.05	
0.005	
—	
—	Control

FIGURE 8

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 92/09127

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl.5 C 07 C 245/24 C 07 D 213/77 A 61 K 31/15
A 61 K 31/44

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl.5	C 07 C C 07 D

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	EP,A,0170951 (DEUTSCHES KREBSFORSCHUNGSZENTRUM) 12 February 1986 see whole document ---	1,17,21
A	BE,A, 514033 (COMPAGNIE FRANCAISE DES MATIERES COLORANTES) 6 March 1953 see claims; examples ---	1-9
A	US,A,3947247 (KOHRMAN ET. AL.) 30 March 1976 see claims; examples --- -/-	1,17,21

° Special categories of cited documents : [10]

"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	Date of Mailing of this International Search Report
11-02-1993	23. 03. 93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	HELPS, I.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	JOURNAL OF MEDICINAL CHEMISTRY vol. 27, no. 3, March 1984, WASHINGTON US pages 357 - 403 K.VAUGHAN ET. AL. 'Studies of the mode of action of antitumour triazenes.6. 1-aryl-3 -(hydroxymethyl)-3-methyltriazenes: synthesis, chemistry and antitumour properties.' see page 359, column 1, compound no. 18 ---	1,3,4, 17,21
A	CHEMICAL ABSTRACTS, vol. 86, no. 25, 20 June 1977, Columbus, Ohio, US; abstract no. 188927k, V. POCHINOK ET. AL. 'Tautomerisation of triazenes' page 527 ;column 2 ; see abstract & UKR. KHIM. ZH. (RUSS. ED.) vol. 43, no. 2, 1977, pages 180 - 3 ---	1
A	CHEMICAL ABSTRACTS, vol. 67, no. 5, 31 July 1967, Columbus, Ohio, US; abstract no. 27572k, Y.B. VILENSKII ET. AL. 'Photographic emulsion study of triazenes. I. aliphatic, fatty aromatic and aromatic triazenes.' page 2608 ;column 2 ; see abstract & ZH. NAUCH. PRIKL. FOTOGR. KINEMATOGR. vol. 12, no. 1, pages 34 - 42 ---	1
P,X	WO,A,9117753 (THE SECRETARY, U.S. DEPARTMENT OF COMMERCE) 28 November 1991 see page 1 - page 5; claims 1,7; examples -----	1,6,17, 21

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/09127

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

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

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 21 and 23-32 are drawn to a method of treatment of the human or animal body by therapy (Rule 39.1(iv)PCT), the search has been carried out and ~~based~~ on the alleged effects of the compounds.

2. ☐ Claims Nos.:
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, specifically:
Claims 16,18,20 and 22 do not fall within the scope of claim 1. R1 and R2 were not defined in claim 1; search was carried out for R1 and R2 = M. No synthetic examples are described for compounds of claims 12 or 13 or indeed for any compound which lies within the scope of claim 1. It is not clear

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

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.

2. ☐ As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ 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 claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/

what protction is really sought by this application (Art. 6 PCT)

Claims searched incompletely: 1-9,12,13,15,17,19,21,23-32
Claims not searched : 10,11,14,16,18,20,22

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 9209127 SA 66654

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 16/03/93
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