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**NOTICE OF ENTITLEMENT**

We, Bayer Corporation, of 800 Dwight Way, Berkeley, California 94701-1986, United States of America, the Applicant in respect of Application No. 14772/95 state the following:

1. Bayer Corporation is the Nominated Person in respect of the application.
2. The actual inventors of the invention, the subject of the application, are:  
Milton, B. Dobkin  
Paul K. Ng  
George B. Dove
3. The Nominated Person, Bayer Corporation, is entitled to the grant of a patent in respect of the application because Miles Inc. derived title to the invention from the actual inventors by way of assignment, and Miles Inc. subsequently changed its name to Bayer Corporation.
4. The Nominated Person is entitled to rely upon the basic application listed on the patent request form because (i) the Nominated Person is the Applicant in respect of the basic application; and (ii) the basic application was the first application made in a Convention country in respect of the invention the subject of the application.

Dated this 1st day of September, 1997.

By Its Patent Attorneys  
**DAVIES COLLISON CAVE**

  
**MARK ROBERTS**



A U S T R A L I A

Patents Act 1990

P/00/001 - Section 29

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**AMENDED**

PATENT REQUEST : STANDARD PATENT

We, being the person(s) identified below as the Applicant, request the grant of a patent to the person identified below as the Nominated Person, for an invention described in the accompanying standard complete specification.

Full application details follow:

[71/70] Applicant/Nominated Person:  
**Bayer Corporation**  
of 800 Dwight Way, Berkeley, California 94701, UNITED STATES OF AMERICA

[54] Invention Title:  
**Use of Azoles as Virucidal Agents in Solutions of Biologically Active Proteins**

[72] Name(s) of actual inventor(s):

[74] Address for service in Australia:  
**DAVIES COLLISON CAVE**, Patent Attorneys,  
of Level 10, 10 Barrack Street, Sydney, 2000, Australia. Attorney Code: CA

BASIC CONVENTION APPLICATION(S) DETAILS:

[31] Application No.	[33] Country	Code	[32] Date
08/226,593	UNITED STATES OF AMERICA		11 April 1994

DATED this 1st day of September, 1997

Bayer Corporation  
By Their Patent Attorneys  
**DAVIES COLLISON CAVE**

  
**MARK ROBERTS**

AUSTRALIA

SEE AMENDED PATENT REQUEST

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FILED: 2 | 9 | 97



PATENT REQUEST : STANDARD PATENT

I/We, being the person/s identified below as the Applicant, request the grant of a patent to the person/s indicated below as the Nominated Person/s, for an invention described in the accompanying standard complete specification.

Full application details follow.

[71] [70] Applicant/s and Nominated Person/s:  
Miles, Inc.,  
of 4th & Parker Streets, BERKELEY, California, 94701, UNITED STATES OF AMERICA

[54] Invention Title:  
Use of azoles as virucidal agents in solutions of biologically active proteins

[72] Name/s of actual inventor/s: (optional)

[74] Address for service in Australia:  
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BASIC CONVENTION APPLICATION/S DETAILS:

[31] Appln No.:	[33] Country:	Code:	[32] Date:
08/226,593	UNITED STATES OF AMERICA	US	11 April 1994

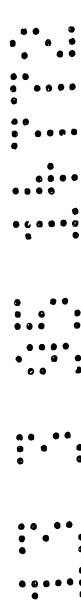
DATED this NINTH day of MARCH 1995

Miles, Inc.  
By Patent Attorneys  
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*Peter Stearne*

Dr. PETER STEARNE, FIPAA

Fee: \$ 280.00





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(12) PATENT ABRIDGMENT (11) Document No. AU-B-14772/95  
(19) AUSTRALIAN PATENT OFFICE (10) Acceptance No. 687153

(Modified Examination)

(54) Title  
USE OF AZOLES AS VIRUCIDAL AGENTS IN SOLUTIONS OF BIOLOGICALLY ACTIVE PROTEINS

International Patent Classification(s)  
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(72) Inventor(s)  
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(74) Attorney or Agent  
DAVIES COLLISON CAVE , GPO Box 3876, SYDNEY NSW 2001

(57) Claim

1. A method of inactivating both enveloped and non-enveloped viruses in an aqueous solution of therapeutic, biologically active proteins selected from the group consisting of coagulation factors and antibodies comprising the step of contacting the solution with an azole selected from the group consisting of imidazole, histidine, 2-imidazolidinone, 1H-imidazole-4-ethanamine under conditions sufficient to result in a reduction of at least 2 logs virus titers and recovery of greater than 50 % of biological activity of the proteins.

AUSTRALIA

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ORIGINAL  
COMPLETE SPECIFICATION  
STANDARD PATENT

Applicant(s):

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SYDNEY NSW 2000

Invention Title:

Use of azoles as virucidal agents in solutions of biologically active proteins

The following statement is a full description of this invention, including the best method of performing it known to me:-

BACKGROUND OF THE INVENTION

Field: This disclosure is concerned generally with methods of inactivating viruses in solutions of biologically active proteins and, specifically, with the use of azoles to inactivate viruses in an aqueous solution of biologically active therapeutic proteins.

Prior Art: The importance of eliminating viral infectivity in therapeutic products has long been recognized. This is especially true in the case of biologically active products derived from human blood or, more recently, from cell cultures used to make products of biotechnology (e.g., recombinant DNA products and monoclonal antibodies).

In considering virucidal agents for biologically active proteins, the primary goals are to assure complete virucidal action while not adversely affecting the biological activity of the protein. These goals require consideration of such variables as the protein itself, the nature of its activity and/or activity site, the virucidal agent, the importance and/or ease of its removal after use, and variables of the treatment itself, such as time, temperature and concentration.

Heat treatment alone can be used for virucidal treatment of some proteins (e.g., pasteurization at 60°C). However, it is difficult in many cases to avoid loss of biological activity or utility when heat alone is used.

To avoid some of the disadvantages or activity losses resulting from the use of heat alone, various chemical agents have been used or proposed as virucidal agents for biologically active proteins. See, for example, U.S. Patent No. 5,071,650, to G. Dove, M. Dobkin and M. Shearer, disclosing the use of alcohols under specific conditions.

The utility of nonexplosive organic solvent/detergent mixtures for the preparation of viral vaccines, and, more recently, for inactivation of endogenous viruses in preparations of biological products derived from human plasma, have been limited by conditions of use (e.g., pH). One virucidal compound used with biologically active proteins at neutral pH is tri-n-butyl phosphate (TNBP). See, for example, U.S. Patent No. 4,540,573 to A. Neurath and B. Horowitz, disclosing the use of TNBP to inactivate lipid-enveloped viruses. See also U.S. Patent No. 5,110,910 to G. Tsay disclosing the use of TNBP as a virucide under controlled pH, conductivity and protein concentration. Unfortunately, current virucides typically inactivate mainly lipid-enveloped viruses and have not been shown to be effective against a hardier class of viruses which lack lipid envelopes.

Various azole compounds have been used as fungicides (e.g., see U.S. Patent 5,006,513 to Hector, et al.), but we are unaware of any suggestion to use them as virucides in solutions of biologically active proteins, especially against non-lipid containing viruses. We now have found that azoles provide a unique ability to inactivate both enveloped and non-enveloped viruses in aqueous solutions while preserving the biological activity of proteins such as immunoglobulins. Details of our method are described below.

SUMMARY OF THE INVENTION

Our method of inactivating both enveloped and non-enveloped viruses in an aqueous solution of biologically active, therapeutic proteins comprises contacting the solution with an azole, under conditions sufficient to assure the substantial reduction of both classes of viruses (2 or more logs virus titer reduction for each class) without adversely affecting the biological activity of the proteins (i.e., greater than 50% recovery of activity). Virucidal activity may be enhanced by the presence of detergents. The primary functional group responsible for inactivation is the pentameric azole ring, which may be modulated by changes to the ring or by additional functional groups attached to the ring. A preferred azole is imidazole, preferably used with a detergent as described below.

SPECIFIC EMBODIMENTS

Azoles have been found to demonstrate virucidal activity with both enveloped and non-enveloped viruses, as illustrated below. As used herein, the terms azole or azole analogs or azole derivatives refer to compounds having a pentameric azole ring and which have virucidal activity under conditions of temperature, pH and concentration which do not adversely affect the biological activity of a protein of interest such as a therapeutic protein.

## MATERIALS AND METHODS

### Chemical Agents

Azoles used were: imidazole, alpha-amino-1H-imidazole-4-propanoic acid (histidine), 2-imidazolidinone (ethylene urea), 1H-imidazole-4-ethanamine (I4EA). Azoles, detergents (e.g., polysorbate 80, Triton X-100), and buffer salts were reagent grade and obtained from Sigma Chemicals, St. Louis, Mo. Polysorbate 80 is an oleate ester of sorbitol and its anhydrides copolymerized with approximately 20 moles of ethylene oxide for each mole of sorbitol and sorbitol anhydrides. Triton X-100 is a trademark for octoxynol.

### Virus

#### Enveloped:

Vesicular stomatitis virus (VSV), Indiana strain, obtained from the Finnish Red Cross, is a Rhabdovirus (RNA). Vaccinia virus, Lederle strain, ATCC VR-118, is a Poxvirus (DNA). Sindbis is a Togavirus (RNA). Herpes simplex virus type 1 (HSV-1) is a Herpesvirus (DNA).

#### Non-enveloped:

Encephalomyocarditis virus (EMC) is a murine picornavirus (RNA). Poliovirus type 2 is a human picornavirus (RNA). Reovirus type 3 virus is a reovirus (RNA). Derivation of viruses was reported previously (see, Lembach, et al., Current Studies in Hematology and Blood Transfusion, Basel, Karger, 1989; 56:97-108).

### Virus Assay

All viruses except Sindbis were titrated under standard conditions on monolayers of VERO cells grown in 24 well plates using 4 wells per dilution. Titers are expressed in terms of tissue culture



infectious doses as a 50% end-point per mL (TCID<sub>50</sub>/mL). Sindbis virus was titrated in similar fashion on monolayers of BHK-21 cells under standard conditions assessing cytopathic effects.

### Proteins

Factor VIII (FVIII), a coagulation factor administered to hemophiliacs, was derived from recombinant baby hamster kidney cells grown in suspension culture. This recombinant FVIII product (rFVIII) is described in EP 160,457 in the name of D.J. Capon, et al.

Monoclonal antibodies (human) of class G (m-IgG, anti-tumor necrosis factor, cell line ATCC Accession No. HB9736, were derived from Epstein-Barr virus-transformed human B lymphocytes grown in suspension culture. IgG was purified to greater than 98% by ion exchange and size exclusion chromatography. These antibodies are described in EP 351,789 in the name of H. Kuo.

Fibrinogen was purified from human plasma by the Cohn-Oncley process. Human serum albumin was purified from human plasma by the Cohn-Oncley process (Fraction V). See, for example, Cohn, E.J., L.E. Strong, W.L. Hughes, D.J. Mulford, J.N. Ashworth, M. Melin and H.L. Taylor. "Preparation and Properties of Serum and Plasma Proteins. IV. A System for the Separation into Fractions of the Protein and Lipoprotein Components of Biological Tissues and Fluids." J. Am. Chem. Soc. 68, 459 (1946).

Protein recovery was evaluated by A280, radial immunodiffusion, and FPLC-Superose 6 (Pharmacia fast protein liquid chromatography by size exclusion). FVIII was assayed by a modified method of

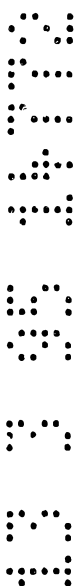
Langdell (Langdell, R.D., Wagner, R.H., Brinkhouse, A. "Effect of anti-hemophiliac Factor on One-Stage clotting Test," J. Lab. Clin. Med. 41: 637-647, 1953) based on activated partial thromboplastin time (APTT).

Treatment Protocol

Protein solutions were seeded with a 1/100 dilution of stock virus to minimize the effects of virus-containing medium. A volume ofazole solution was added to give a final desired concentration. Treated and control samples were incubated with intermittent gentle mixing under the specified conditions (time, temperature). Samples were removed at appropriate intervals and titrated immediately for residual infectious virus.

RESULTS

Studies were conducted to determine the types of azoles exhibiting virucidal activity. As seen in Table 1, a wide variety of azoles are effective in reducing VSV titer. The primary functional group resulting in virus inactivation is the pentamericazole ring, which may be modulated by changes to the ring or by additional functional groups attached to the ring.



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Table 1

Inactivation Kinetics of VSV in FVIII containing a variety of azoles at 40°C.

Azole Concentration	Imidazole 0.02M	Imidazole 0.2M	Histidine 0.2M	2-Imidazolidinone 0.2M	I4EA 0.2M
Time (hrs)	Virus Titer Remaining				
0	(7.5*)	(7.75)	(8.25)	(7.75)	(8.25)
1	--	6.25	7.0	>7.5	7.25
3	--	3.75	4.5	7.0	5.5
4	--	2.5	3.0	5.75	5.0
5	--	<1.5	2.5	5.75	4.25
6	5.75	<1.5	2.75	5.25	3.5

( ): untreated

\*:  $\text{LOG}_{10} \text{TCID}_{50} / \text{ML}$

--: not tested

Studies were conducted to determine the types of viruses susceptible to inactivation by azoles. Table 2 lists conditions necessary to provide substantial virus reduction (2 or more logs reduction) for a variety of enveloped and non-enveloped viruses.

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Table 2

Inactivation Parameters for a Variety of Enveloped and Non-Enveloped Viruses in FVIII.

Virus	VSV	Enveloped Viruses			Non-Enveloped Viruses		
		HSV-1	Sindbis	Vaccinia	Polio-2	EMC	REO-3
Treatment Time (hr)	0.25	0.25	<1	<1	7	4	7
Temperature (C)	15	15	15	40	40	40	40
Imidazole (M)	0.02	0.02	0.02	0.2	0.2	0.2	0.2
Triton X-100 (%)	0.05	0.05	0.05	0.1	0.1	0.1	0.1
Titer (initial)	8.0*	6.75	8.25	6.25	6.5	6.5	7.75
Titer (final)	<1.5	<1.5	<1.5	<1.5	4.5	1.5	4.25
Reduction	>6.5	>5.25	>6.75	>4.75	2.0	5.0	3.5

\*:  $\text{LOG}_{10}\text{TCID}_{50}/\text{ML}$

Detergents may enhance virucidal activity. Table 3 lists several examples, demonstrating specific conditions of increased activity or lack thereof.

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Table 3

Effect of Detergents and Temperature on Virucidal Activity of Imidazole.

Virus	Sindbis		EMC		
Temperature (C)	15		15	40	
Imidazole Conc'n. (M)	0.02		0.2	0.2	
Triton X-100 Conc'n. (%)	none	0.05	none	0.1	none
Time (hr)	Virus Titer Remaining				
0	8.25*	8.25*	6.5	6.5	6.5
0.5	--	2.0	--	6.0	5.7
1	8.0	<1.5	6.25	5.75	5.7
2			5.75	6.25	3.25
5			6.25	6.25	<1.5

\*:  $\text{LOG}_{10}\text{TCID}_{50}/\text{ML}$

--: not tested

A variety of proteins in aqueous solutions are not substantially affected by treatment with azoles. Recovery may vary, depending on the protein. For example, FVIII is a very large, labile protein. Conditions permitting substantial virus inactivation without loss of activity are uncommon. Table 4 defines kinetics for FVIII.

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Table 4

Protein Recovery in the Presence of Imidazole Under a Variety of Conditions.

Protein	FVIII	FVIII	FVIII	IgG	Fibrinogen
Imidazole Concentration (M)	0.02	0.2	0.2	0.5	0.2
Temperature (C)	40	30	40	40	40
Time	Protein Recovery (% of initial)				
0	100	100	100	100	100
1		91	85		
2			92		
3		83	86		
4		83	79		
5		84	78		
6		86	77		
7	>95	81	75		
24	>95			>95	>95

DISCUSSION

Speculation on how imidazole works is as follows:

Each virion consists of a protein coat which has many clusters of protein subunits. Subunits are stabilized in solution by metal ions and may dissociate when the metal concentration is reduced. The binding of protein subunits to metal ions is facilitated by the electron-donating side chains of residues such as histidine. A complex is formed between a metal ion and the epsilon nitrogen from the imidazole ring of a histidine residue on the protein. In virus-containing solution, free imidazole competes with the imidazole on the protein subunits. As metal ions bind to the free imidazole, the protein subunits become unstable and the virus coat is opened, thereby destroying the native structure of the virion. The phenomenon is also dependent on the concentration of protein (e.g., FVIII) in solution, further supporting that imidazole in solution competes with the imidazole on the protein.

Given the above disclosure it is thought variations will occur to those skilled in the art. Thus, the above examples should be construed as illustrative and the invention disclosed here should be limited only by the following claims.

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The Claims defining the invention are as follows:

1. A method of inactivating both enveloped and non-enveloped viruses in an aqueous solution of therapeutic, biologically active proteins selected from the group consisting of coagulation factors and antibodies comprising the step of contacting the solution with an azole selected from the group consisting of imidazole, histidine, 2-imidazolidinone, 1H-imidazole-4-ethanamine under conditions sufficient to result in a reduction of at least 2 logs virus titers and recovery of greater than 50 % of biological activity of the proteins.
2. The method of claim 1 wherein the azole is imidazole.
3. The method of claim 1 comprising the additional steps of including a detergent selected from polysorbate 80 and Octoxynol.
4. The method of claim 3 wherein the detergent is Octoxynol.
5. The method of claim 1 wherein the biologically active protein is Factor VIII.
6. The method of claim 1 wherein the biologically active protein is fibrinogen.

DATED this 3rd day of March 1997



MILES INC  
By Its Patent Attorneys  
DAVIES COLLISION CAVE

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ABSTRACT OF THE DISCLOSURE

Soluble azoles in aqueous solutions can be used as virucidal agents for biologically active protein preparations.

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