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(54) Title: RICIN INHIBITORS AND METHODS FOR USE THEREOF		
(57) Abstract		
<p>Ricin A-chain is an N-glycosidase that attacks ribosomal RNA at a highly conserved adenine residue. Crystallographic studies show that not only adenine and formycin, but also pterin-based rings can bind in the ricin active site. For a better understanding of the recognition mode between ricin, and adenine-like rings, the interaction energies and geometries were calculated for a number of complexes. Shiga toxin, a compound essentially identical to the protein originally isolated from <i>Shigella dysenteriae</i>, has an active protein chain that is a homologue of the ricin active chain, and catalyzes the same depurination reaction. The present invention is drawn to identifying inhibitors of ricin and Shiga toxin, using methods molecular mechanics and ab initio methods and using the identified inhibitors as antidotes to ricin or Shiga toxin, or to facilitate immunotoxin treatment by controlling non-specific cytotoxicity.</p>		

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5 RICIN INHIBITORS AND
 METHODS FOR USE THEREOF

BACKGROUND OF THE INVENTION

Federal Funding Legend

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Field of the Invention

15 The present invention relates generally to protein
chemistry. More specifically, the present invention relates to
the identification of inhibitors of the heterodimeric cytotoxin
ricin, using computer modeling of the ricin active site as a
template in structure-based drug design. Moreover, the present
20 invention relates to the use of the identified inhibitors as
antidotes to ricin or to facilitate immunotoxin treatment by
controlling non-specific cytotoxicity. In addition, Shiga toxin,
a compound essentially identical to the protein originally
isolated from *Shigella dysenteriae*, has an active protein chain
25 that is a homologue of the ricin active chain, and catalyzes the
same depurination reaction. Thus, the present invention is drawn
additionally to identifying inhibitors as antidotes to Shiga
toxin.

Description of the Related Art

30 Ricin is a potent heterodimeric cytotoxin easily
isolated from the seeds of the castor plant, *Ricinus communis*.

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The protein consists of a lectin B chain, which can bind cell surfaces and is linked by disulfide bonds to an A chain (RTA), which enzymatically depurinates a key adenine residue in 28 S rRNA (Endo and Tsurugi, J. Biol. Chem., 262:8128-30 (1987)).

5 Ricin is an extraordinarily toxic molecule that attacks ribosomes, thereby inhibiting protein synthesis. Ricin has an LD₅₀ of approximately 1 µg/Kg body weight for mice, rats, and dogs and is ten times more potent against rabbits (Olsnes, S. and Pihl, A., "Toxic Lectins and Related Proteins," The Molecular
10 Action of Toxins and Viruses, pp. 52-105 (1982)). The toxic dose for humans is likely to be in the µg/Kg range which ranks it among the most toxic substances known. The protein has been used extensively in the design of therapeutic immunotoxins. In these constructs, ricin, RTA, or a related toxin is chemically or
15 genetically linked to an antibody to form a so-called "magic bullet", which can target preferentially those cell lines carrying antigenic markers recognized by the antibody (Frankel, A.E., ed., Immunotoxins, Kluwer academic publishers, Boston (1988)).

20 Ricin also has been used as a poison agent. The protein gained notoriety when it was used in the famous "umbrella tip" assassination of Georgy Markov and was also used in an unsuccessful attempt to poison the famous Soviet dissident Alexander Solzhenitsyn. Recently, ricin was prepared by a
25 militant anti-tax group which planned to poison IRS personnel; they were arrested and convicted.

Given the above, there is interest to identify or design potent inhibitors of ricin. These inhibitors could, in

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principle, be used to facilitate immunotoxin treatment by helping to control nonspecific cytotoxicity, or could be used as antidotes to poison attacks. Recently there has been interest in structure-based drug design--that is, using the knowledge of protein structure to identify enzyme inhibitors. The most common paradigm for this overall process has been called an "iterative protein crystallographic algorithm" by Appelt et al., J. Med. Chem. 34:1925-34 (1991), where the design of inhibitors for thymidylate synthetase is described. The main concept of the approach is that the protein active site is used as a template to design or to identify complementary ligands. The identified putative ligands are ranked and tested kinetically. Promising inhibitor candidates are bound to the protein target and analyzed crystallographically for comparison with the proposed model. Additionally, alterations are made in the inhibitor to improve binding and a new round of tests is carried out on the altered compound.

A number of laboratories have used variations on this protocol to design efficacious inhibitors. For example, the search program DOCK (see Kuntz et al., J. Mol. Biol. 161:269-88 (1982)) was used to predict that the known anti-psychotic drug haloperidol would bind to the HIV protease (DesJarlais et al., Proc. Natl. Acad. Sci USA 87:6644-48 (1990)). Crystallographic studies together with computer aided search methods also were used in the design of inhibitors of purine nucleoside phosphorylase. The program GRID (Goodford, P. J., J. Med. Chem. 28:849-57 (1985)) has been used to design two very successful inhibitors of influenza virus. Certain chemical substitutions to the sialic acid substrate were predicted to be energetically

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favorable, based on interactions with the known X-ray structure of the enzyme. Subsequent binding assays revealed K_i values as low as 0.2 nM. These compounds not only inhibited neuraminidase but retarded viral replication in cultured cell and animal models as well.

The X-ray structure of ricin has been solved (Montfort et al., *J. Biol. Chem.* 262:5398-03 (1987)), refined to 2.5 Å (Rutenber et al., *Proteins* 10:240-50 (1991)), and described in detail (Katzin et al., *Proteins* 10:251-59 (1991); and Rutenber and Robertus, *Proteins* 10: 260-69 (1991)). The structure of RTA expressed in *Escherichia coli* has been resolved to 2.3 Å resolution for monoclinic crystals (Mlsna et al., *Prot. Sci.* 2:429-35 (1993)), and recently to 1.8 Å resolution for a tetragonal form (Weston et al., *J. Mol. Biol.* 244:410-422 (1994)).

The X-ray model of RTA allowed identification of a number of amino acids which were hypothesized to be important for substrate binding and for the depurination mechanism; these residues include Glu 177, Arg 180, Trp 211, Tyr 80 and Tyr 123. Site-directed mutagenesis of the cloned RTA gene has been used to examine the relative significance of these residues (see, e.g., Schlossman et al., *Mol. Cell. Biol.* 9:5012-21 (1989); Frankel et al., *Mol. Cell. Biol.* 10:6257-63 (1990); Ready et al., *Proteins* 10:270-78 (1991); and Kim and Robertus, *Protein Engineering* 5:775-79 (1992)). In addition, Monzingo and Robertus, *J. Mol. Biol.* 227:1136-45 (1992), carried out an X-ray analysis of substrate analogs in the RTA active site, examining FMP, adenylyl guanosine (ApG) and guanylyl adenosine (GpA). The structure of important substrate and analog bases, together with the

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numbering scheme used in energy minimizations, is shown in Figure 6.

Several closely-related mechanisms of action have been proposed which incorporate elements of both the structural and kinetic analyses. It is likely that the susceptible adenine base binds between tyrosines 80 and 123 while forming specific hydrogen bonds with the backbone carbonyl and amido nitrogen of Val 81 and with the carbonyl of Gly 121. In the hydrolysis, the leaving adenine is at least partially protonated by Arg 180, and Glu 177 may stabilize a putative oxycarbonium transition state or, more likely, act as a base to polarize the attacking water.

Shiga toxin, a compound essentially identical to the protein originally isolated from *Shigella dysenteriae*, has an A chain which is activated by proteolysis, generating an active A1 enzyme and an A2 fragment, which remains bound until a disulfide bond linking them is reduced. The active A1 chain (STA1) is a homologue of RTA, and catalyzes the same depurination reaction.

E. coli strains can carry the gene for Shiga toxin, and this renders them pathogenic. Human infection lead to hemorrhagic colitis and hemolytic-uremic syndrome--commonly referred to as "hamburger disease"--a severe and often fatal form of food poisoning. It is difficult to control outbreaks of hamburger disease because antibiotics tend to lyse the bacteria, releasing the destructive toxin into the system, aggravating tissue damage and internal bleeding. An effective inhibitor of STA1 would be a powerful adjunct to treatment of hemorrhagic colitis and hemolytic-uremic syndrome.

The Shiga toxin gene has been cloned and engineered to express the enzyme. A comparison of the amino acid sequences of

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RTA and STA show clearly that they are homologues. An energy-minimized model of STA1 was constructed (Deresiewicz et al, Biochemistry 31:3272-80 (1992)), and it was noted that key residues conserved among plant Ribosome Inactivating Protein
5 (RIP) enzymes are conserved in STA1 (Katzin et al., Proteins 10:251-59 (1991)).

Until the present invention, no inhibitors for RTA or STA1 had been identified. Even FMP, which is known to bind RTA, is not an effective inhibitor of the RTA. Thus, the prior art
10 is deficient in identifying compounds which are effective inhibitors of ricin. The present invention fulfills this long-standing need and desire in the art.

SUMMARY OF THE INVENTION

The present invention provides compounds that are
15 effective inhibitors of the cytotoxic proteins ricin or Shiga toxin.

In one object of the present invention, there is provided a compound effective for inhibiting ricin, said compound able to act within an active site of RTA and having an aromatic
20 heterocyclic molecular core, wherein said aromatic heterocyclic molecular core resembles an adenine moiety in size and shape and is derivatized with at least one polar substituent such that said polar substituent interacts in said active site of RTA. In another embodiment of this object of the invention there is
25 provided a compound wherein said polar substituent is an amine group able to donate hydrogen bonds to a carbonyl oxygen of Val 81 or Gly 121. As another embodiment of this object of the present invention, there is provided a compound wherein said inhibitor further comprises at least one pendant group which

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binds an amino acid adjacent to said active site. In addition, said inhibitor further may comprise at least one moiety which reacts with a shallow channel in said RTA chain.

In another object of the present invention, there is provided a compound effective for inhibiting ricin, said compound being able to act within an active site of RTA, having nonpolar interactions with a side chain of an amino acid in said active site selected from the group Tyr 80, Ile 172 or Tyr 123, and having polar interactions with a side chain of an amino acid in said active site, selected from the group of carbonyl oxygens of Gly 121 or Val 81, backbone amides of Val 81 or Tyr 123, or atoms on side chains of Arg 180, Tyr 80, Tyr 123 or Asn 78. In addition, an embodiment of the present object provides an inhibitor further comprising at least one nonpolar moiety which interacts with side chains from Trp 211, Leu 45, Val 256, Tyr 257 or Thr 77 of said RTA chain. Alternatively, said inhibitor may possess at least one polar moiety which interacts with the carbonyl oxygens of Thr 77 or Tyr 257, or side chains from Asn 47 and Arg 258 of said RTA chain, or further comprise at least one polar moiety which interacts with an amino acid from a second pocket of said RTA chain. In addition, said inhibitor may interact in a nonpolar fashion with side chains from amino acids selected from the group of Tyr 80, Val 82, Phe 57, Thr 77 and Arg 56.

In yet another object of the present invention, there is provided a compound effective for inhibiting ricin, said compound selected from the group of pteric acid, neopterin, pterin (3) tautomer and guanine (4) tautomer.

In an additional object of the present invention,

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there is provided a compound effective for inhibiting Shiga toxin, said compound able to act within an active site of STA1 and having an aromatic heterocyclic molecular core, wherein said aromatic heterocyclic molecular core resembles an adenine moiety
5 in size and shape and is derivatized with polar substituents such that said polar substituents interact in said active site of RTA.

An additional object of the present invention, there is provided a compound effective for inhibiting Shiga toxin, said compound selected from the group of pteric acid, neopterin, pterin (3) tautomer and guanine (4) tautomer.
10

Additionally, the present invention provides a method for identifying a compounds effective for inhibiting ricin, comprising the steps of performing at least one technique from the group of molecular modeling, crystallography, and energy
15 minimization; protein synthesis assay; and phage display, and a method for identifying a compounds effective for inhibiting Shiga toxin, comprising the steps of performing at least one technique from the group of molecular modeling, crystallography, and energy minimization; protein synthesis assay; and phage display.

Another object of the present invention is to provide the compounds pteric acid, neopterin, pterin (3) tautomer and guanine (4) tautomer as pharmaceutical compounds as antidotes to ricin or to facilitate immunotoxin treatment by controlling non-specific cytotoxicity.
20

Other and further aspects, features, and advantages of the present invention will be apparent from the following description of the presently preferred embodiments of the invention. These embodiments are given for the purpose of disclosure.
25

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BRIEF DESCRIPTION OF THE DRAWINGS

The appended drawings have been included herein so that the above-recited features, advantages and objects of the invention will become clear and can be understood in detail.

5 These drawings form a part of the specification. It is to be noted, however, that the appended drawings illustrate preferred embodiments of the invention and should not be considered to limit the scope of the invention.

Figure 1 shows the inhibition of ricin A chain (RTA) by pterioic acid. Pterioic acid (squares) has a minor inhibitory effect on the protein synthesis using *Artemia salina* ribosomes.

Protein synthesis is reduced to about 20% in the presence of 1.5 nM RTA (circles) but toxin action is inhibited by increasing doses of pterioic acid. The inset replots the level of RTA activity as a function pterioic acid concentration.

Figure 2 shows the electron density for pterioic acid complexed to RTA. This is OMIT density based on $F_o - F_c$ coefficients and phases from the protein model in which Tyr 80 has been displaced to form the normal substrate binding site.

20 The difference density baskets are contoured at the 3s level. The refined position of PTA is shown superimposed on the difference density.

Figure 3 illustrates the interactions between pterioic acid and RTA. Hydrogen bonds are shown as dashed lines with the lengths indicated.

Figure 4 shows the superposition of the formycin and pterin rings in the RTA active site. Carbon atoms are solid, nitrogen atoms have a light pattern and oxygens have a darker pattern. Hydrogen bonds between PTA and RTA are dashed lines and

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those between FMP and RTA (Monzingo and Robertus, J. Mol. Biol. 227:1136-45 (1992)) are dotted.

Figure 5 shows the binding of neopterin in the RTA active site. Hydrogen bonds are shown as dashed lines and the lengths are indicated.

Figure 6 shows the structure and numbering of important substrate analogs for RTA. Nucleosides based on adenine, guanine, and formycin are linked to ribose at ring position 9. Pterin derivatives discussed in this specification are modified at position 6.

Figure 7 shows the crystallographically-observed complex of RTA and FMP. The figure shows the substrate recognition cleft occupied by FMP, an AMP analog. Hydrogen bonds are shown as dashed lines, bonds within RTA are lighter than those within FMP. Carbon and hydrogen atoms are black, oxygen is a light pattern, and nitrogen and phosphorous are a darker pattern.

Figure 8 shows the chemical structures and partial charges for ring compounds examined in this study. Charges were derived from ab initio minimization and used in subsequent docking experiments with RTA.

Figure 9 shows the bonding interactions predicted between RTA and various ring compounds. Partially charged compounds were docked into RTA and minimized in SYBYL. Hydrogen bonds are shown as dashed lines, bonds within RTA are lighter than those within the ligand; carbon and hydrogen atoms are black, oxygen is a light pattern, and nitrogen is a darker pattern. The figure shows RTA interactions with a) formycin ring, b) adenine, c) pterin(1) tautomer, d) pterin (3) tautomer,

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e) guanine(4) tautomer, and f) AHA (3-amino-4-hydroxybenzoic acid).

Figure 10 shows the interactions between RTA and two forms of pterin. Panel A shows the hydrogen bonds and charge distribution of RTA interacting with pterin(1) and B shows the interactions with pterin(3). Strong bonds (2.6 to 2.8 Å) are dashed lines and weaker bonds (3.0 to 3.3 Å) are dotted. Partial charges are indicated by the δ symbol.

Figure 11 shows the correlation of ligand structure for the design of RTA inhibitors. Panel A shows the observed binding of nucleotides like FMP and AMP. Panel B shows the binding of pterin based inhibitors, and Panel C shows the limitations of guanine nucleotides as ligands.

DETAILED DESCRIPTION OF THE INVENTION

It will be apparent to one skilled in the art that various substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention.

As used herein, the term "ricin" refers to a heterodimeric cytotoxic protein isolated from *Ricinus communis*, or a protein expressed in a heterologous system from the ricin gene. Ricin contains a lectin B chain (RTB) and an N-glycosidase A chain (RTA).

As used herein, the term "RTA substrate specificity pocket" refers to the portion of the RTA active site that binds adenine. The term "RTA active site" refers to that portion of RTA which binds the rRNA substrate. The active site is large, generally polar, and interacts with the highly-charged rRNA. The

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purine ring of the rRNA adenine rests between the side chains of tyrosines 80 and 123, and four polar protein groups in the active site (O121, O81, NH81 and NH1 of Arg 180) make hydrogen bonds with the substrate.

5 As used herein, the term "secondary pocket" refers to the portion of the RTA molecule that is a fairly large and generally nonpolar cavity of the opposite side of Tyr 80 from the adenine binding site.

 As used herein, the term "shallow channel" refers to
10 the portion of the RTA molecule that forms a sort of rut, runs from the specificity pocket along its surface, and is flanked by Arg residues (213, 258, 48 and 56, moving away from the catalytic site).

 As used herein, the term "inhibitor" refers to a small
15 molecule which binds to RTA and retards its enzyme action.

 As used herein the term "immunotoxin" refers to refers to a chemical or genetic linkage between an antibody with a binding preference for a target cell type, and a toxic protein such as ricin or RTA.

20 As used herein the term "non-specific cytotoxicity" refers to the intoxication of cells which are not targeted by an immunotoxin.

 As used herein the term "pteroic acid" or "PTA" refers to 4-[[[(2-amino-1,4-dihydro-4-oxo-6-pteridiny)]
25 methyl]amino]benzoic acid.

 As used herein, the term "formycin monophosphate" or "FMP" refers to an AMP analog in which the adenine ring is replaced by a pyrasolopyrimidine ring (that is, the N9 of adenine is replaced by C and C8 is replaced by N).

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As used herein, the term "neopterin" refers to 2-amino-6-(1,2,3-trihydroxypropyl)-4(3H)-pteridone.

As used herein, the term "guanine (4) tautomer" refers to the guanine tautomer in which hydrogen is bonded to N3 and not
5 to N1.

As used herein, the term "inhibitor design" refers to a process of identifying inhibitors or improving the properties of a known inhibitor.

As used herein, the term "molecular modeling" or
10 "computer modeling" refers to fitting a model ligand compound, such as an inhibitor candidate, into the active site space of a protein model and adjusting the models according to chemical principles to simulate their interactions; this is usually done with a computer display system.

As used herein, the term "X-ray structure" refers to
15 a model of a protein or protein•ligand complex which is based on experimental data obtained by X-ray diffraction from crystals of the protein or complex.

As used herein, the term "SYBYL" refers to a computer
20 program distributed by TRIPOS Corporation which can be used to model proteins or complexes, and also can be used to calculate the energy of interaction between atoms or groups, based on various approximations of chemical laws.

As used herein, the term "6-31g** basis set" refers to
25 a mathematical function used by the program SYBYL to approximate the distribution of electrons within atomic orbitals.

As used herein, the term "X-PLOR" refers to a suite of computer programs written by Axel Brunger and used routinely to

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adjust models to their lowest energy state, consistent with the observed experimental X-ray diffraction data.

As used herein, the term "energy minimization" refers to adjusting the atoms or chemical groups within a model so that the new model assumes the lowest energy--that is, the most likely conformation of the model, based on approximations to chemical laws.

As used herein the term "OMIT" refers to a kind of electron density map based on the observed X-ray amplitude data for a protein and phases calculated from a partial model.

As used herein the term " $F_o - F_c$ " refers to the coefficients used to compute a kind of electron density map commonly called a "difference Fourier," and used to show those parts of a protein or complex which have changed as a result of some action, such as, but not limited to, inserting a ligand.

Ricin is a potent cytotoxin which has been used widely in the construction of therapeutic agents such as immunotoxins.

Recently it has been used by governments and underground groups as a poison. There is interest in identifying and designing effective inhibitors of the ricin A chain (RTA). The present invention utilizes computer-assisted searches to identify compounds that bind in the RTA active site, which normally recognizes a specific adenine base on rRNA. Kinetic assays indicated that pteronic acid (PTA) could inhibit RTA activity with an apparent K_i of 0.6 mM, and a 2.3 Å crystal structure of the complex revealed the mode of binding. In the same way that PTA was identified as an inhibitor, other compounds have been assayed in the method of the present invention. The present invention is directed to compounds that are effective inhibitors of the

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cytotoxic protein ricin and methods for screening for additional inhibitors.

In addition, a molecular model of STA1 based on RTA has been proposed. The X-ray structure of the Shiga toxin B chain has been solved, as has the intact (A1A2)B₅ toxin, which showed that the proposed model was sound. Respective matching of the RTA and STA1 active site finds Arg 180, the key catalytic residue in RTA, corresponds to Arg 170 in STA1, Glu 177 with Glu 167, Tyr 80 with Tyr 77, Tyr 123 with Tyr 114, and Trp 211 with Trp207. The X-ray structure of the Shiga toxin holotoxin (A1A2B₅) has been solved (Fraser, et al., Nature Struc. Biol. 1:59-69 (1994), confirming the general similarity of RTA and STA1.

The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion.

EXAMPLE 1

Preparation Of Recombinant RTA And Protein Synthesis Assay

Recombinant RTA was prepared as described by Ready et al., Proteins 10:270-78 (1991). Recombinant RTA was prepared as described by Chaddock and Roberts, Protein Engineering 6:425-31 (1993). Briefly, 50 ml cultures of E. coli JM101 harboring plasmid pUTA (Ready et al, Proteins 10:270-78 (1991)) was grown overnight at 37°C, then used to inoculate 500 ml 2YT media and grown for 2 hours at 30°C. Expression of RTA was induced by addition of 50 µl 0.5M isopropyl-1-thio-β-galactoside (IPTG) and incubation for 4 hours at 30°C. Cells were harvested by centrifugation, resuspended in 30 ml 5 mM sodium phosphate (pH

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6.5), and broken with a French pressure cell. Following centrifugation to remove cell debris, the supernatant was loaded onto a 100 ml carboxymethyl-sepharose (Pharmacia) column equilibrated in the same buffer. Unbound protein was washed from the column with 1500 ml buffer followed by 200 ml buffer containing 100 mM sodium chloride. RTA was eluted with a linear 500 ml 0.1-0.3 M sodium chloride gradient. Peak RTA-containing fractions were pooled and stored at 4°C at approximately 1mg/ml. Typical yields were 40-50 mg RTA/l media for wild type RTA.

RTA activity was measured by its ability to inhibit protein synthesis in an in vitro protein synthesis assay using *Artemia salina* ribosomes, as described previously by Ready et al, *Biochem. Biophys. Acta* 740:19-28 (1983) and Ready et al, *Proteins* 10:270-78 (1991). Thirty-four pmoles of *Artemia salina* ribosomes were incubated in a volume of 120 µl for 5 minutes at 25°C with inhibitor (ptericoic acid) ranging in concentration from 0.062 mM to 1.67 mM, with or without 1.5 mM spermine. The reaction was stopped by the addition of 400 nmoles of anti-RTA antibody. 100 µl of a protein synthesis mix containing ¹⁴C-Phe and wheat germ extract was added to all the tubes. After a 15 minute incubation at 25°C, the reaction was stopped by the addition of 5% TCA. The mixture was heated to 90°C for 10 minutes, then passed through a Millipore nitrocellulose filter. The filters were dried and counted by liquid scintillation counting. Inhibitor candidates were judged by their ability to disrupt RTA action against the *Artemia* ribosomes.

Pterin compounds including pterin-6 carboxylic acid,

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neopterin, pteric acid, and folic acid were purchased from Sigma Chemical Company (St. Louis). Concentrations were evaluated spectroscopically, assuming the same ultraviolet spectrum as folic acid, that is $\epsilon_{283} = 25,000$. Because of the limited solubility of the pterins, the ribosome inhibition assays and controls were carried out at pH 9.

EXAMPLE 2

Crystallography And Molecular Modeling

Crystals of RTA were grown in the monoclinic form (see Robertus et al., J. Biol. Chem. 262:19-20 (1987); and Mlsna et al., Prot. Sci. 2:429-35 (1993)) or in a tetragonal form (Weston et al., J. Mol. Biol. 244:410-22 (1994)). Three-dimensional diffraction data were collected on a San Diego Multiwire Systems area detector (see Hamlin, Methods in Enzymology 114:416-52 (1985)) with a Rigaku RU-200 X-ray source operating at 50 kV, 110 mA with a graphite monochromator. Data were collected using the method of Xuong et al., J. Appl. Cryst. 18:342-50 (1985), and reduced and evaluated using the University of California, San Diego (UCSD) software system (Howard et al., Methods in Enzymology 114:453-72 (1985)). Rotation and translation searches, as well as crystallographic refinement of the RTA inhibitor complex (energy minimization and simulated annealing), were carried out using the X-PLOR package (Brünger, Crystallographic refinement by simulated annealing, Crystallographic Computing 4: Techniques and New Technologies, Oxford: Clarendon Press, pp. 126-140. (1988)). Molecular modeling was carried out using FRODO (see Jones, FRODO: A graphics fitting program for macromolecules, Computational

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Crystallography, Oxford: Oxford University Press, pp. 303-317 (1982)), running on an Evans and Sutherland PS390.

Molecular modeling programs CHEMX and SYBYL were purchased from Chemical Design Ltd. (Oxon, England) and Tripos Inc. (St. Louis) respectively. Interaction energies were calculated with the Tripos force field. The energy minimization was terminated when a 0.05 energy gradient value was reached using the Powell algorithm as implemented in SYBYL. A distance dependent and a constant function (dielectric constant = 1.5) were used for dielectric effects, with the expectation that the distance dependent function can simulate the internal protein dielectric, while the constant function simulates the solvent effect on the protein. Non-bonded cutoff was 8.0 C. The net atomic charges used in minimization were from Kollman method (Weiner et al., J. Am. Chem. Soc., 106:765-84 (1984)) for RTA and from ab initio calculation (6-31g** basis set) for ligands; these were calculated on CRAY YMP and COIL located at the National Institutes of Health.

EXAMPLE 3

20 Energy Minimization

According to the ab initio minimization, the hybridization of an amino N atom connected to an aromatic ring is variable depending on its environment. Table 1 lists the deviation from the ring plane of two hydrogens on such N atoms. It shows that some N atoms are sp² type, and some are combinations of sp² and sp³ types. If the deviation sum from the aromatic plane is larger than 30 degrees, the N atom was dealt with as sp³ type in the molecular mechanics calculation.

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Table 1: The deviation sum (degree) of hydrogens of amino N atom from aromatic ring plane according to ab initio (6-31g**) minimization

structure	deviation
formycin	41.1
adenine(1)	0.7
adenine(2)	44.0
Pterin(1)	37.1
pterin(2)	0.8
pterin(3)	36.9
pterin(4 ion)	0.1
guanine(1)	38.1
guanine(2)	27.8
guanine(3)	32.1
guanine(4)	41.3
2,4-diamino-pteridine	*
3-Amino-4-hydroxybenzoic acid (ion)	55.7
S2(ion)	0.0

* There are two amino N atoms, on which all hydrogens are in the aromatic ring plane.

In the energy minimization protocol, the energies for the crystal complexes of ricin with FMP (Monzingo, and Robertus, J. Mol. Biol. 227:1136-45 (1992)) and pteric acid were minimized first. Next, these structures were reminimized after FMP and pteric acid were replaced by formycin and pterin. Then the formycin or pterin was replaced by other structures one by one. The newly-formed complexes also were fully minimized by the

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criteria mentioned above for obtaining the interaction energies between ricin and ligands. The interaction energy was obtained by subtracting the unliganded ricin energy and ligand energy (in its binding conformation) from the complex energy. Some of the structures studied were small and not very different from formycin, adenine or pterin. It was assumed that these small structures causes only small changes in the RTA conformation and there is no requirement to perform molecular dynamics simulation for conformation searching.

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EXAMPLE 4

Identification Of Pteric Acid As An Inhibitor

A query was constructed using the geometric and bonding parameters of the observed FMP ligand, and a search of the NCI data base was made with CHEMX. Among the compounds predicted to have hydrogen bond donors and acceptors in an orientation favorable for RTA interaction was the pterin derivative called pteric acid (PTA). SYBYL calculations suggested that PTA might bind better than FMP. Interaction enthalpies, calculated assuming one negative charge on each ligand, were -106 Kcal/mole for pteric acid, as compared with -89 Kcal/mole for FMP. It is important to note that the interaction enthalpies do not consider such terms as configurational entropy nor the effect of solvent interactions, and thus are not meant to represent the free energy of binding the ligand to the protein. Rather, the calculation is intended to serve as a rough guide to comparing the likely affinities of related compounds for the target enzyme.

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To determine if pteric acid was indeed an inhibitor of RTA, it was tested in the protein synthesis assay; because of

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the limited solubility of PTA, the experiments and controls were carried out at pH 9. Figure 1 shows protein synthesis on 300 nM *Artemia* ribosomes in the presence of varying concentrations of pteronic acid, and in the presence or absence of 1.5 nM RTA. The figure shows that pteronic acid has a minor inhibitory effect on protein synthesis itself--a 1.7 mM concentration of pteronic acid inhibits protein synthesis about 20%. The inset panel replots the data as the fraction of possible RTA activity versus the concentration of pteronic acid. It shows that 0.6 mM pteronic acid reduces RTA action by 50% and defines the effective K_i for the inhibitor.

Several efforts were made to observe crystallographically the binding of pteronic acid to RTA. The strongly diffracting tetragonal crystals are grown at pH 4.5 and would not bind the inhibitor either when soaked in a saturated solution or when cocrystallized. However, monoclinic crystals at pH 9 were made 20 % saturated with pteronic acid and soaked for seven days. Although the crystals cracked initially, over the soaking time most of the cracks annealed. Diffraction showed that the complex was not isomorphous with the native. Native cell parameters are $a = 42.6$, $b = 68.1$, $c = 50.2 \text{ \AA}$ and $\beta = 112.9^\circ$ (Robertus et al., J. Biol. Chem. 262:19-20 (1987)). The pteronic acid complex crystals have the following parameters: $a = 39.0$, $b = 64.4$, $c = 49.4 \text{ \AA}$ and $\beta = 108.0^\circ$.

Three dimensional data were collected to 2.3 \AA including 68,262 observations of 10,999 reflections; reflection intensities scaled with $R = 6.5 \%$. Since the crystals were not isomorphous with the native, the RTA•PTA complex was solved using

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molecular replacement methods. The monoclinic RTA was used as a model for a rotation search using the program X-PLOR. Data between 10 and 4 Å showed no rotation. A translation search was then executed with X-PLOR. The translation search revealed a translation of 0.65 Å along x, and of 2.82 Å along z. The initial positioning was completed using the rigid body refinement option of X-PLOR; the R factor to 3 Å resolution was 0.36.

A difference map with coefficients (Fo-Fc) was calculated and showed a mixture of positive and negative peaks within the active site. These peaks could be interpreted as the binding of PTA in the area previously seen to bind FMP. The pterin ring occupies the same site as the formycin ring of FMP or the adenine ring of the ApG dinucleotide. This requires that the Tyr 80 side chain rotate roughly 45° to stack with the aromatic surface of the pterin, in a way similar to that seen for FMP (see Monzingo and Robertus, J. Mol. Biol. 227:1136-45 (1992)). The Tyr 80 position in the model was adjusted and a second (Fo-Fc) map computed. This OMIT map is shown in Figure 2. There is clear electron density for the pterin moiety of the inhibitor; the benzoic acid density is weaker but still allows that group to be readily positioned. PTA was built into the density and the model subjected to four successive rounds of energy minimization and simulated annealing using X-PLOR. Between each round of refinement, the resolution was increased by 0.2 Å beginning at 3.0 Å and ending at 2.3 Å. Another difference map was calculated and 45 waters were added to the structure. After a final round of energy minimization and isotropic temperature factor refinement, a final R-factor of 17.9% was calculated for all data to 2.3 Å. Isotropic

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temperature factor refinement showed the pterin ring, with average B values of 11, was more rigidly held than the benzoic acid moiety where average B values were 26. The occupancy of the pteric acid refined to 1.0, as compared to 0.5 seen previously
5 for FMP (see Monzingo and Robertus, J. Mol. Biol. 227:1136-45 (1992)).

Figure 3 illustrates the interactions between PTA and the active site of RTA. The pterin ring binds in roughly the position of the substrate adenine, sandwiched between the rings
10 of Tyr 80 and Tyr 123. For comparison, Figure 4 shows a superposition of the formycin ring of FMP (Monzingo and Robertus, J. Mol. Biol. 227:1136-45 (1992)) and the pterin ring of PTA.

The 2.6 Å distance between N1 of pterin and the carbonyl oxygen of Gly 121 suggests that the pterin is stabilized in a tautomeric
15 form with a hydrogen on N1 (shown in Table 2) and this can be donated to the carbonyl group. Note that the 2-amino group of pterin also donates a hydrogen bond to the carbonyl oxygens of Gly 121 as well as to Val 81; this roughly mimics the role of the 6-amino group on an adenine substrate. In the same way, N3 of
20 pteric acid resembles N1 of adenine, receiving a hydrogen bond from the amido N of Val 81. The adenine substrate receives a hydrogen bond at N3 from Arg 180, and indeed this is the likely route of substrate protonation in the catalytic mechanism. Pteric acid receives two hydrogen bonds from Arg 180, at the 4-
25 oxo and N5 positions.

The benzoate moiety of PTA bends around the side chain of Tyr 80 and probably makes some nonpolar interactions with it. In fact, the benzoate ring appears to bind on the surface of a pocket--a site hypothesized by Monzingo and Robertus, J. Mol.

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Biol. 227:1136-45 (1992), to be a second recognition site which may accommodate the guanine base of a natural rRNA substrate.

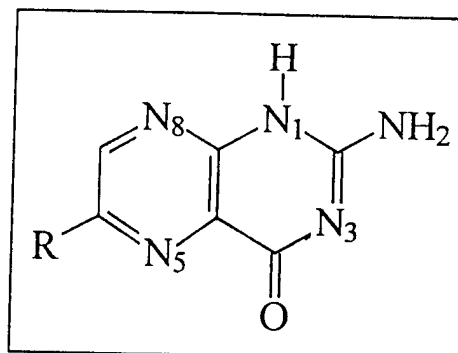
Two hydrogen bonds are made to the benzoate, one between the carboxylate and Asn 78, and one to a water molecule which also
5 bonds Arg 258. Although the pterin moiety of PTA makes strong and specific interaction within the adenine recognition site, it seems apparent that the benzoate moiety of PTA is not optimized for interactions within the second site.

EXAMPLE 5

10 Screening Additional Compounds

Following the analysis of PTA, structural and kinetic studies were carried out on a number of other pterin-based compounds, as shown in Table 2. These compounds differ essentially in the size of the group attached to pterin at
15 position 6.

Table 2: Pterin Based Compounds as Potential Inhibitors of RTA



Substituent	Name	RTA Inhibitor	X-ray Model
COO^-	6-carboxy pterin	No	No
	neopterin	$K_i \approx 2 \text{ mM}$	2.5 Å
	Pteric acid (PTA)	$K_i = 0.6 \text{ mM}$	2.5 Å
	Folic acid	No	No

The parent compound, pterin, is shown at the top of the table with a general substituent group, R, at position 6. The compounds in the table are modified at that position with the groups shown. The compound name and status as an inhibitor is also indicated.

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Neopterin is pterin derivatized at the six position with propane triol. It was observed in the protein synthesis assay to be a modest inhibitor of RTA, with a $K_i > 2$ mM (data not shown). Monoclinic crystals of an RTA complex were obtained by cocrystallization; they were isomorphous with the native, and diffracted to 2.5 Å resolution. 58,838 observations of 9,030 reflections to 2.5 Å resolution were collected ($R_{merge} = 6.6\%$), the data reduced, and difference Fourier maps calculated. The pterin ring bound in the site previously seen to bind the formycin ring of FMP (Monzingo and Robertus, *J. Mol. Biol.* 227:1136-45 (1992) and the pterin ring of PTA as described above. As with PTA and FMP, the side chain of Tyr 80 was displaced by the ligand. Neopterin was positioned by hand, including the propane triol moiety, and X-PLOR used to adjust position of the ligand and the protein in a simulated annealing refinement; the R factor refined to 19.5 % for all data. Coordinates for both the PTA and neopterin complexes have been submitted to the Brookhaven Protein Data Bank.

The binding of neopterin to RTA is shown in Figure 5. The orientation of the pterin ring is similar to that seen for PTA. One difference is the bonding of Arg 180 to the inhibitor. It bonds to the 4-oxo group of neopterin, but does not bond to N5 as occurs in the PTA complex. Instead, a second bond is formed with the proximal hydroxyl of the propane triol moiety. Associated with this rearrangement is a 7° rotation of the pterin ring. The other atoms of the propane triol moiety of neopterin make no interaction with RTA. Since the propane triol moiety of neopterin is much smaller than the corresponding

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substituents in PTA, it does not interact with Tyr 80 in the same way and appears to lack the van der Waals contribution to binding which might be expected in PTA. This is consistent with energy calculations using SYBYL, which show interruption enthalpies between RTA and PTA to be -106 Kcal/mole and those between RTA and neopterin to be -73 Kcal/mole. Kinetic inhibition data, although not definitive, also suggest PTA is a better inhibitor and more likely to exhibit tighter binding. The stronger binding of PTA compared with neopterin argues that design of a pterin-based moiety derivatized at the 6 position which makes a strong and specific interaction with RTA is the preferred approach in creating an efficient inhibitor.

Crystals of RTA•neopterin were isomorphous with native RTA, but crystals of RTA•PTA were reproducibly not isomorphous; RTA•PTA crystals exhibited a contraction of about 3 Å along both the a and c axes. A least squares superpositioning of the two complexes showed very few significant structural differences between them. The largest changes were at the N and C termini, both far removed from the substrate (inhibitor) binding site. The chemical differences between the two inhibitors are centered on the size and charge of the moiety derivatizing pterin at position 6. However, there are no direct interactions of the bound inhibitor with any crystallographically-related molecules. Furthermore, the protein conformation itself is very similar in these two active sites, and so it does not appear that inhibitor binding induces protein changes which then are involved directly in crystal packing. It may be that inhibitor binding triggers a series of very subtle changes which propagate

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throughout the protein and cause a rearrangement of packing, although this seems unlikely. It is also possible that saturating the solution with PTA has an unspecified solvent effect which tends to dehydrate the crystals and causes them to shrink. Except for this phenomenon, the binding of the two inhibitors seems to have roughly the same effect on RTA conformation, moving the Tyr 80 side chain. Further, inhibitor design can use this observed binding as a foundation and that novel inhibitor models can be fit to this template without undue concern about predicting protein responses.

Neither pterin-6-carboxylic acid nor folic acid acted as inhibitors of RTA within the limits of their solubility. Efforts to soak the compounds into monoclinic crystals, even at saturating conditions, and cocrystallization efforts failed to produce stable complexes for X-ray analysis.

Given knowledge of the binding of PTA and neopterin it was predictable that neither pterin-6-carboxylic acid nor folic acid would bind to RTA. Refinement and energy minimization using SYBYL suggested that the interaction enthalpy for pterin-6-carboxylic acid, with one negative charge, was low (-64 Kcal/mole) compared with PTA or neopterin. Inspection of the hypothetical binding, assuming that the pterin moiety assumes the position seen in PTA, suggests that the 6 carboxylate is near the Glu 177 residue of RTA. Charge repulsion may therefore prevent binding of this simple compound.

Folic acid does not inhibit RTA, nor does it bind to RTA crystals. Folic acid resembles PTA except that the benzoic acid moiety is derivatized with long and negatively charged glutamic acid. Electrostatic mapping of the RTA surface shows

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the mouth of the second recognition site, beyond the area binding
the PTA benzoate, is generally negative due to the presence of
residues like aspartates 75, 96, and 100. This negative charge
likely repels the folic acid group, driving it into solution and
5 limiting binding compared with PTA.

Figure 8 shows the net atomic charges, derived from ab
initio minimization with the 6-31g** basis set, on additional
ligands examined by the methods of the present invention. Table
3 lists the molecular energies of these compounds in atomic
10 units, au, where 1 au = 627.5 Kcal. Table 3 also lists the
interaction energies between RTA and each ligand calculated from
molecular mechanics methods (Tripos force field). The
interaction energies are broken into Van der Waals and
electrostatic components. The active site binding geometries for
15 the energy minimized complexes with formycin, adenine, pterin(1),
pterin(3) and guanine(4), computed by molecular mechanics, are
presented in Figure 9.

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Table 3: molecular energy (au) from ab initio minimization and interaction energy (Kcal/mol) between ricin and these structures from molecular mechanics minimization

5		Interaction energy (Kcal/mol)			
molecular	structure	energy (au)	vdw	electrostatic	total
	formycin	-464.4888712	-19.2	-24.5	-43.7
	adenine(1)	-464.5361520	-18.0	-19.4	-37.4
10	adenine(2)	-464.5211196	-19.0	-20.5	-39.5
	Pterin(1)	-577.2721321	-23.1	-22.0	-45.1
	pterin(2)	-577.2702506	-22.8	-21.8	-44.6
	pterin(3)	-577.2624667	-22.6	-31.8	-53.4
	pterin(4, ion)	-577.6574933	-22.9	-24.6	-47.5
15	guanine(1)	-539.4125625	-18.7	-16.1	-34.8
	guanine(2)	-539.4129359			
	guanine(3)	-539.4055644	-21.8	-20.0	-40.8
	guanine(4)	-539.4005964	-20.8	-33.0	-53.8
	2-hydroxy formycin	-539.3674887	-18.7	-29.1	-47.8
20	2,4-diamino-pteridine	-557.4495408	-23.2	-18.9	-42.1
	aha(ion)	-547.6530542	-19.6	-48.5	-68.1
	emodin	-948.2356142	-30.0	-44.0	-74.3
	rhodizonic acid	-677.4887203	-19.6	-25.5	-45.1
	lumazine	-597.0937652	-17.5	-22.5	-40.0
25	s1	-597.1110054	-18.0	-25.7	-43.7
	S2(ion)	-466.8918064	-14.5	-41.1	-55.6
	S3	-450.2572027	-15.7	-24.1	-39.8
30	vdw	--- van der Waals interaction energy (kcal/mol)			
	aha	--- 3-Amino-4-hydroxybenzoic acid			

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Based on energy minimization, the formycin ring structure forms 7 hydrogen bonds in the RTA binding site (Fig 5 9a). The ligand has an interaction energy with RTA of -43.7 Kcal/mol. The calculated orientation of the free formycin generally is similar to the ring seen crystallographically in the RTA•FMP complex (Figure 7). However, note the free ring is shifted slightly so that an extra hydrogen bond is donated by the backbone NH of Tyr 123 to N8 of formycin. Also, the guanidinium 10 of Arg 180 donates two bonds instead of one to N3. The lack of the ribose ring allows the Arg 180 side chain to reorient in this way.

Adenine is the natural substrate for RTA and is 15 recognized by the enzyme, at least when it is part of a GAGA loop context. For the two major adenine tautomers (see Figure 8), the molecular energy of adenine(1) is 9.4 Kcal/mol (0.015 au) lower than adenine(2), suggesting it is the normal solution form. Adenosine nucleotides are derivatized at N9 and so are frozen 20 into the tautomer(1) form. Figure 9b shows the interaction of adenine(1) with RTA. Table 3 shows that the free adenine(2) interacts slightly more strongly with RTA than does adenine(1).

The main reason for this is that in adenine(2) the hydrogen on N7 can be donated to O of Gly 121. Weston et al., J. Mol. Biol. 25 244:410-422 (1994), found that AMP was hydrolyzed in a tetragonal form of RTA crystals leaving only adenine bound. The occupancy was low, however, and it was not possible to assess the tautomeric form of adenine. In the design of inhibitors,

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derivatives of either form would seem equivalent.

The interaction energies between RTA and the four underivatized pterin tautomers (one is in the ion form) were minimized and are listed in Table 3. Pterin(1), the most stable
5 tautomer in aqueous solution, has an interaction energy around -45 Kcal/mol (Figure 9c), as strong as for formycin. The exocyclic 6-amino (N12 in Figure 1), donates hydrogen bonds to O of Gly 121 and Val 81, reminiscent of the amine group in formycin and adenine. The 4-oxo atom (O11 in Figure 6) receives
10 bonds from NH of Val 81 and the O_y of Ser 176, while N8 receives a bond from the NH of Tyr 123 and N5 receives two bonds from Arg 180. Because the interaction energy is as strong as for formycin, it is reasonable to assume that pterin(1) may bind weakly in the RTA active site. However, pterin(1) forms fewer
15 and generally weaker hydrogen bonds with RTA than does pterin(3), shown in Figure 9d. A comparison of the binding of the two pterin forms is also diagrammed in Figure 10, where strong hydrogen bonds are shown as dashed lines and weaker ones as dotted lines.

It shows the pterin(1) complex forms three strong and four weak
20 bonds, while pterin(3) complex forms six strong and two weak bonds. In particular, loss of the hydrogen from N3 of pterin(1) allows that atom to receive a bond from the NH of Val 81 (2.8 Å), and acquisition of a hydrogen at N1 allows it to donate a bond to the O of Gly 121 (2.6 Å). Consistent with this, the
25 interaction energy for pterin(3) is about 8 Kcal/mole more favorable than for pterin(1). The X-ray data are consistent with the binding of pterin(3). The observed distance between pterin N3 and the backbone N of Val 81 is 3.2 Å, impossible if both

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have hydrogens on them. Also the distance between O of Gly 121 and N1 and the exocyclic N of pterin are 2.6 and 2.8 Å respectively, suggesting two strong hydrogen bonds. It is apparent that the increased stability of binding tautomer(3) to RTA compensates for the cost of shifting from the more stable tautomer(1).

It is apparent from the interaction energies that the strong binding of PTA (see Yan, et al., "Structure-based Identification of a Ricin Inhibitor" (submitted to JMB) (1996)) results not simply from interactions with the pterin ring, even its tautomer(3) form, but also from additional interactions with the benzoate-containing group linked at C6. Compared with PTA, the free pterin group also orients in a slightly different fashion based on molecular mechanics calculations. In particular, a hydrogen bond can be formed from O_y of Ser 176 to the 4-oxo of free pterin, whereas this is much weaker (3.4 Å) in the PTA complex. However, Ser 176 should be exploited in the design of inhibitors.

The binding of pterin(3) to RTA may result from simple equilibrium considerations. Pterin(3) is much less common in solution than form 1, but once bound the equilibrium shifts to produce more form 3. Alternatively, it may be that the more stable pterin(1) initially recognizes the RTA active site. Then, a shift of the protons from N3 to N12 and a shift from N12 to N1 take place, creating the pterin(3) tautomer; Figure 10 illustrates this process. Tautomerization may be triggered by the repulsive forces between the positive charges on Arg 180, Ser 176, and Val 81 of the protein, and the positive charge on N3 of

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pterin(1). At the same time there is an electrostatic attraction to move protons to N12 which is surrounded by negative charges from Gly 121 and Val 81.

The success of binding pterins to RTA raises questions about binding guanine derivatives, which have many of the same chemical features as pterins. Ab initio calculation shows that guanine(1), the form seen in nucleotides, is a low energy tautomer, while guanine(4) is the highest. From the interaction energies in Table 3, it can be seen that the guanine(4) interacts most strongly with RTA, as strongly as pterin(3). This putative binding to RTA is shown in Figure 9e, where the carbonyl (6-oxo) of guanine(4) accepts two hydrogen bonds from the guanidinium group of Arg 180, while other groups interact with RTA in a fashion similar to pterin(3). The stability of guanine(4) compared to guanine(1) arises from the same considerations as were described for pterin(3) binding compared to pterin(1). Interaction energy calculations suggest guanines are reasonable inhibitor candidates. Attempts to bind 2-amino-6,8-dihydroxypurine (8-hydroxy guanine) to RTA were unsuccessful although the solubility of such compounds is limited and crystallization conditions may not allow strong binding to occur.

Since the interaction energies of guanine compounds is computed to be high compared to adenine, one might ask about the specificity of RTA for adenine. Figure 11 integrates information about the bonding of adenines, pterins, and guanines. It shows that guanine nucleotides cannot bind to the RTA active site for trivial steric reasons. Panel A shows the binding of adenine-like nucleotides such as FMP and ApG seen crystallographically. The R group would be a ribose linking the base into a larger rRNA

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molecule. Panel B shows the observed binding of pterin based ligands; PTA and neopterin are derivatized at position 6, shown by the R. Panel C shows that the guanine(4) base can assume an electronic configuration like pterin, but compared with adenine, it is flipped over. Note that the R group at position 7 now clashes with the protein. Non-nucleoside guanine compounds might still be expected to bind well to RTA if they are modified at positions 8 or 9. Compounds like 2-amino-6,8-dihydrooxypurine did not bind to RTA, but are poorly soluble and it simply may be that under crystal conditions these compounds cannot achieve high enough concentrations to bind well to the protein. On the other hand, it should be noted that, unlike pterin(1), the interaction energy of guanine(1) with RTA is quite low, about -34.8 Kcal/mol, compared to -45.1 Kcal/mol. If a model as shown in Figure 10 is appropriate, the initial recognition of guanine may be very unlikely.

In addition to the purine and pterin bases, a number of other compounds were analyzed as potential RTA inhibitors. The interaction energy of 2,4-diamino-pteridine with RTA is -42.1 Kcal/mol (Table 3). This energy is substantially weaker than for pterin because the 4-amino hydrogens repulse the guanidinium group of Arg 180 and N1 repulses the carbonyl O of Gly 121. Rhodizonic acid has 4 carbonyl groups and 2 hydroxyl groups; it might seem to be flexible enough to form a variety of interactions with RTA. Its interaction energy is about -45 Kcal/mole, similar to formycin, and yet it failed to bind to RTA crystals. It should be noted however, that the interaction energy for FMP is -89 Kcal/mole and the additional interactions outside the specificity pocket may be important to judging the

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effectiveness of binding. In a similar fashion, lumazine, S1, and S3 (Table 3 and Figure 8) interact with RTA at only modest strength, implying that they are unlikely to be good inhibitors of RTA themselves, but can serve as the parent compound for other inhibitor classes.

Table 3 shows the interaction energies of AHA and emodin with RTA to be relatively strong, based on molecular mechanics. Although they are predicted to bind well to RTA, they have quite different interaction geometry compared with adenine, pterin, and formycin. The putative binding of AHA is shown in figure 9f. Attempts to soak these highly colored compounds into RTA crystals failed, showing that a strong interaction energy is not sufficient to assure ligand binding.

In summary, the RTA specificity site is formed largely by the backbone of residues Gly 121, Asn 122, Tyr123 and Val 81, and by side chains of Arg 180, Tyr 80, Tyr123, Glu177, and Trp 211. The interaction energy from hydrogen bonds is a major factor in determining RTA substrate specificity, and in particular at recognizing one adenine base in a particular rRNA context. The main donors and acceptors of RTA binding site to a base are the carbonyls of Gly 121 and Val 81, the amine of Val 81 and the side chain Arg 180. Obviously, the carbonyls and backbone amides are not flexible. The Arg 180 chain is not flexible either, because it forms an aromatic stack with Trp 211 and is strongly fixed by hydrogen bonds with the carboxylate of Glu177 and the peptide carbonyl of Asn 78. The guanidinium side chain can move only within very limited space, and in the minimizations of the present invention, it always maintains its geometry. Taken together, this suggests that the RTA active site

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has evolved to act as a fairly rigid receptor to bind specific, complementary ring structures bearing a resemblance to adenine.

It is possible to bind compounds, like pterins, which make additional contacts and have stronger interaction energies with the active site. This is an important consideration in inhibitor design. RTA has evolved to recognize an extended rRNA substrate and its strong binding to ribosomes ($K_m \sim 1 \mu M$) arises partly from interactions at sites remote from the catalytic adenine recognition site. It is these areas that may need to be targeted for optimum inhibitor design.

EXAMPLE 6

Phage Display And Inhibitor Design

In addition to query-based search methods, phage display search methods are used to identify peptides which bind strongly to RTA and Shiga toxin. The method is useful in inhibitor/drug design in two ways. First, peptides can be identified that inhibit the toxins directly. Second, the crystal structure of tight-binding peptides likely reveals novel enzyme•ligand interactions since the peptides differ markedly from the heterocyclic systems observed crystallographically. The novel interactions identified are then fed into the query-based searches.

The principles of the phage display method are generally well known (Clackson and Wells, TIBTech 12:173-84 (1994)). M13 are filamentous phage with a major coat protein called P8 and a surface protein P3 involved in infectivity; both proteins have been engineered for phage display. P3 is localized at one end of the phage and is present in only 3 to 5 copies; the

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amino terminus of P3 can accommodate large peptide inserts without interfering with folding or infectibility. A commercial library was obtained from New England Biolabs containing all of the 1.3×10^9 ($=20^7$) random heptapeptides displayed on P3. This is about the upper limit of library size, given current transformation efficiencies for E. coli, and the limits of convenient handling of cell volumes. The manufacturer's simplest protocol for screening suggests attaching RTA to the wells of a microtiter plate via nonspecific hydrophobic interactions.

10 Roughly 10^{11} phage (about 100 copies of each displayed heptamer) were applied and allowed to bind. Those with poor binding were simply rinsed away. The tighter binding phage were then eluted, as suggested, using free RTA. The eluted phage were amplified in E. coli, titered, and used in a second screening round. A total of four rounds were carried out, presumably selecting for stronger binding peptides (the phage were eluted in rounds 3 and 4 using low pH instead of free RTA, as is discussed below). Ten phage from the last round were sequenced over the insert region, using the commercially available sequencing primer. Seven phage showed a consensus tetrapeptide sequence: Ser-(Ser/Thr)-(Leu/Ile)-Pro.

25 Native RTA is presented to the phage library, as opposed to RTA which has been nonspecifically adsorbed to microtiter plates. The standard RTA expression vector used in these experiments has been engineered to include coding for an amino terminal His₆ peptide. The His-tagged protein (his-RTA) is soluble and fully active and has been purified on standard Ni-NTA columns, indicating it binds Ni well. Microtiter plates obtained from QIAGEN Corporation coated with a Ni-NTA matrix binds the

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His-RTA to the wells through the His leader, displaying a fully active enzyme with its active site facing the solvent.

Phage binding to the RTA plates could be eluted with free RTA in rounds 1 and 2, but free RTA could not release phage
5 in round 3. This has been interpreted to indicate that free RTA is not a strong enough competitor to release the peptide ligands and denaturation of RTA with low pH is required to allow phage elution. The implication is that the very strongest ligands were probably not released in the early rounds and were not amplified.
10 Ultimately, peptides were selected with a clear affinity for RTA. Stronger stripping methods, such as low pH are used to identify better ligands.

Once a strongly-binding peptide is identified, it must be synthesized, being acetylated at the N terminus and amidated
15 at the C terminus. The neutralization of the terminal charges minimize the chances of repulsion between the peptide and charged groups in the RTA active site. Once synthesized, the peptide is purified and tested as an inhibitor of RTA (for example, in the protein synthesis assay described in Example 1), and is then
20 diffused into RTA crystals or cocrystallized with RTA for crystallographic examination as described in Example 2.

In addition to the linear-heptapeptide library, a cyclic peptide library may be utilized. A cyclic peptide is characterized by a fixed disulfide bond defining a loop of six
25 random amino acids. The disulfide is flanked by at least one random amino acid on each end. Alternatively, a similar library is used, having four randomized residues in the loop, or having a random hexapeptide loop and four randomized residues flanking the disulfide. Cyclic peptides are advantageous in that they are

-40-

structurally more rigid and constrained, and have greatly reduced flexibility compared to linear peptides. Binding of the corresponding free cyclic peptides to the enzyme target is entropically favorable compared with linear peptides and they
5 tend to have stronger association constants. For example, it has been shown that the affinities of cyclic peptides for streptavidin exceed those of similar linear peptides by 100-1000 fold (Giebel, et al., Biochemistry 34:15430-35 (1995). Further, X-ray structures confirm the notion that the cyclic peptides are
10 more compact and constrained in the active site.

EXAMPLE 7

Inhibitor Design For STA1

Inhibitor design for STA1 follows the paths described in the Examples above for RTA; that is, computer searches,
15 kinetic assays and phage display techniques are utilized. The plasmid pSC25 expresses STA. A stop codon is engineered into this plasmid at a position to produce the 32 kD active STA1 peptide, removing the inhibiting STA2 peptide instead of cleaving it with proteases. The engineered 28 kD protein is expressed and
20 isolated for crystallization experiments. Further, a polyHis sequence is engineered to both the N and C termini of STA1, which expedites purification and allows for isolation of useful crystals since each modified construct represents an independent protein, as far as crystallization is concerned. Such tags attached to
25 RTA did not hamper full enzymatic activity, and it is likely that identical tags will not hamper STA1 enzymatic activity. The work described above has provided a model of STA that is used for structure-based drug design; however, a workable crystal model of STA makes the best use of the iterative crystallographic

-41-

algorithm of drug design.

Expression of the STA1 protein in the engineered pSC25 allows for kinetic tests to be carried out on various inhibitors.

Obviously, due to the similarities between STA1 and RTA, it makes sense to try inhibitors on STA1 that have been identified as being effective on RTA. For example, pteronic acid inhibits RTA, and is tested on STA1. There are, however, differences between RTA and STA1 that potentially are important. Gly 121 is a key residue in the RTA active site, since its carbonyl group receives a hydrogen bond from the 2 amino group of pteronic acid.

The corresponding residue in STA1 is Ser 112, and the disposition of the Ser side chain suggests that it may also interact with pteronic acid. However, the Ser112 hydroxyl can form an additional bond, donating to N8 of the inhibitor, that is not formed by the RTA Gly, suggesting that pteronic acid may bind to STA1 more effectively than it does to RTA.

EXAMPLE 8

Synthetic Approach To Identifying Inhibitors

Once compounds are identified through the above-referenced computer modeling, crystallographic, energy minimization, phage display or protein synthesis assay means, the compounds are synthesized. A recent advance in synthetic organic chemistry involves the multiple simultaneous (multiplex) synthesis of an analogous series of compounds. Rather than performing one to three reactions in a given day, 30-100 reactions are run using an appropriate reaction manifold. The key is the purification of the individual products, which can be facilitated by supporting the fixed reagent on an insoluble polymer allowing simple filtration, followed by cleavage of the

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product from the support.

Alternatively, polymer-supported reagents are used to affect coupling of the two reactants, or to scavenge side-products, thereby converting them to insoluble material, which is easily separated from the reaction mixture by filtration. In yet another alternative, solution-phase chemistry is employed, followed by standard aqueous work-up procedures or selective crystallization of the of the product from the reaction mixture.

A relatively inexpensive apparatus facilitating parallel syntheses and allowing temperature control, variable speed mixing, and the maintenance of an inert atmosphere over each reactor has been devised. Work-up and purification of the multiple reactions run in this apparatus is simplified by the use of a solid-supported scavenger which reacts with excess reagent to form an insoluble product that is easily separated from the reactions mixture by filtration. Between 10 and 100 mg of each product is synthesized. In addition to providing ample sample for biological evaluation, this allows each product's identity and purity to be assayed using NMR techniques.

Scheme 1 below shows one class of parallel synthesis starting with pteronic acid and using carbonyldiimidazole or oxalyl chloride coupling agents or solid-phase synthesis with the pteronic acid group attached through the 2-amino substituent to a polymer support. Scheme 2 shows a second example of multiplex synthesis using 2-pivaloyl-6-formylpterin as the starting material. Condensation of this material with a variety of amines leads to imine intermediates that are reduced to afford the 2-pivaloyl-protected analogs. Purification of these protected analogs is simplified by use of a polymer-supported reducing

-43-

agent, which is removed by filtration. The protected analogs are subjected to mild deprotection conditions and isolated by crystallization.

In parallel with the multiplex synthesis approach, traditional synthetic techniques are employed to prepare analogs of compounds identified as effective in inhibiting RTA or STA1. For example, analogs of pteric acid in which the pterin moiety is replaced with an alternative heterocycle are prepared. Focus is on heterocycles predicted to adopt a tautomeric structure with strong binding to the adenine specificity pocket. The hydrogen binding properties of pterin(3) are present, but do not have to undergo an energetically unfavorable tautomerization. The structure below shows the generalized form of a series of these compounds. In this series, the steric and hydrogen bonding potential about the sulfur is varied systematically by manipulating the oxidation state from the sulfide to sulfoxide to sulfone.

Additional changes in the hydrogen bonding potential are explored by examining both the 1,2,4-benzothiadiazine (I, X-CH) and 1, 2, 4-pyridothisadiazine 1,1-dioxides; the preferred tautomeric form in solution is the 4-H form shown in structure I. An additional advantage to these compounds when compared to pteric acid is increased aqueous solubility. Also, modeling within the RTA specificity site suggests that the polar interaction between the sulfone group ($Y=SO_2$) and Arg 180 is substantially stronger than those made to the 4-oxo group of PTA.

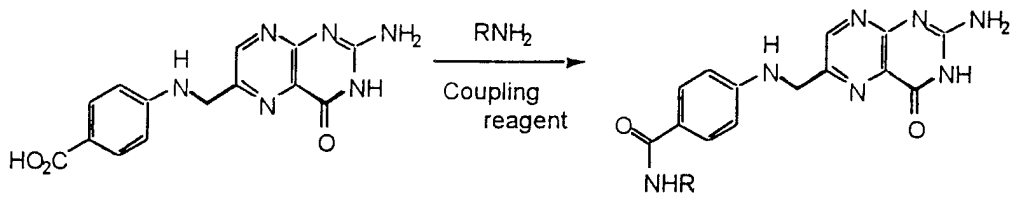
Finally, when developing inhibitors into drugs, it is important to note that compounds identified may bear a strong resemblance to known and approved compounds; for example, the compounds

-44-

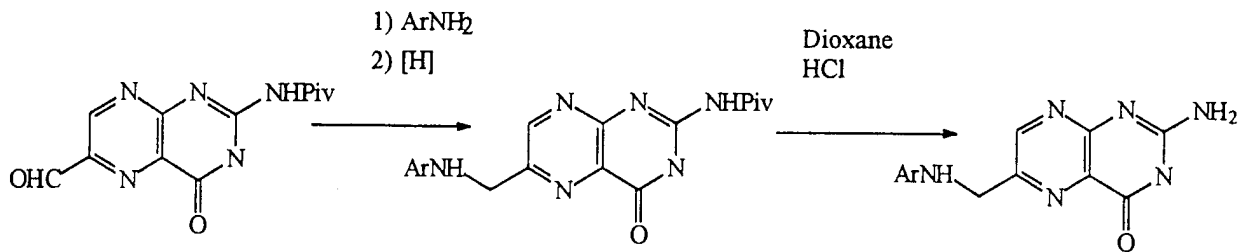
described above resemble benzothiadiazine diuretics like Aldactazine and Diuril.

The synthesis of compounds of general structure I proceeds based upon literature precedence. A general route to
5 2-amino-substituted 1,2,4-benzothiadiazines involving displacement of a 3-methylthio group has been reported by Pirotte, et al, J.Med. Chem. 36:3211-13 (1993); and Tullio et al, Tetrahedron 51:3221-34 (1995) have demonstrated the synthesis of
10 3-amino-substituted[2,3]pyrido-1,2,4-thiadiazines from 3-amino-2-sulfamoylpyridine, as shown in Scheme 3. Although 1,2,4-pyridothisdiazine-1,1-dioxides are known, there have not been reports of 1,2,4-pyridothisdiazines in which the sulfur is not oxidized, or is in the sulfoxide oxidation state. However, Finch et al., J. Org. Chem. 45:3416-21 (1980) reported routes to the
15 corresponding 1,2,4-benzothiadiazines and 1,2,4-benzotthisdiasine 1-oxides, Scheme 4. Adaptation of these rounds allows production of the corresponding pyrido-analogs.

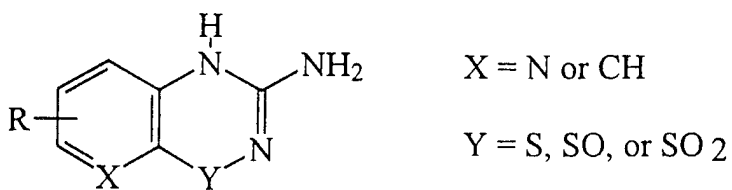
-45-



Scheme 1

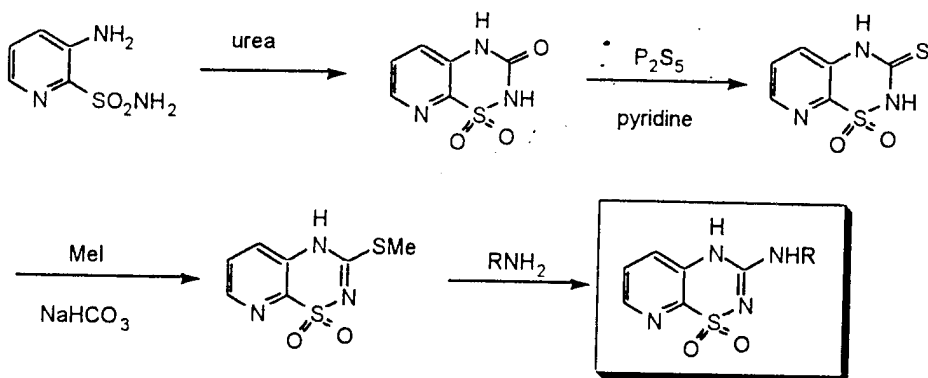


Scheme 2

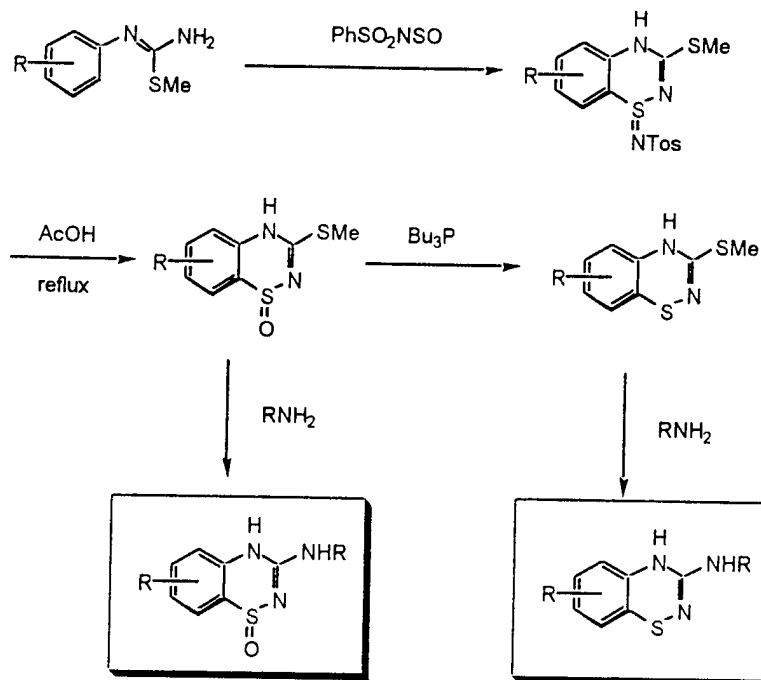


Structure I

-46-



Scheme 3



Scheme 4

-47-

Any patents or publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. Further, these patents and publications are incorporated by reference herein to the same extent as if each individual publication was specifically and
5 individually indicated to be incorporated by reference.

One skilled in the art will appreciate readily that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those
10 objects, ends and advantages inherent herein. The present examples, along with the methods, procedures, treatments, molecules, and specific compounds described herein are presently representative of preferred embodiments, are
exemplary, and are not intended as limitations on the scope of
15 the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention as defined by the scope of the claims.

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Claims

1. A compound effective for inhibiting ricin, said compound able to act within an active site of RTA and having an aromatic heterocyclic molecular core, wherein said aromatic heterocyclic molecular core resembles an adenine moiety in size and shape and is derivatized with polar substituents such that said polar substituents interact in said active site of RTA.

2. The compound of claim 1, wherein said polar substituent is an amine group able to donate hydrogen bonds to a carbonyl oxygen of Val 81 or Gly 121.

3. The compound of claim 1, wherein said inhibitor further comprises at least one pendant group which binds an amino acid adjacent to said active site.

4. The compound of claim 1, wherein said inhibitor further comprises at least one moiety which reacts with a shallow channel in said RTA chain.

5. A compound effective for inhibiting ricin, said compound being able to act within an active site of RTA, having nonpolar interactions with a side chain of an amino acid in said active site selected from the group Tyr 80, Ile 172 or Tyr 123, and having polar interactions with a side chain of an amino acid in said active site selected from the group of carbonyl oxygens of Gly 121 or Val 81, backbone amides of Val 81 or Tyr 123, or atoms on side chains of Arg 180, Tyr 80, Tyr 123 or Asn 78.

-49-

6. The compound of claim 5, wherein said inhibitor further comprises at least one nonpolar moiety which interacts with side chains from Trp 211, Leu 45, Val 256, Tyr 257 or Thr
5 77 of said RTA chain.

7. The compound of claim 5, wherein said inhibitor further comprises at least one polar moiety which interacts with the carbonyl oxygens of Thr 77 or Tyr 257, or side chains from
10 Asn 47 and Arg 258 of said RTA chain.

8. The compound of claim 5, wherein said inhibitor further comprises at least one polar moiety which interacts with an amino acid from a second pocket of said RTA chain.
15

9. The compound of claim 8, wherein said inhibitor interacts in a nonpolar fashion with side chains from amino acids selected from the group of Tyr 80, Val 82, Phe 57, Thr 77 and Arg
20 56.

10. A compound effective for inhibiting ricin, said compound selected from the group of pteric acid, neopterin, pterin (3) tautomer and guanine (4) tautomer.

25 11. A compound effective for inhibiting Shiga toxin, said compound able to act within an active site of STA1 and having an aromatic heterocyclic molecular core, wherein said aromatic heterocyclic molecular core resembles an adenine moiety in size and shape and is derivatized with polar substituents such

-50-

that said polar substituents interact in said active site of RTA.

12. A compound effective for inhibiting Shiga toxin,
said compound selected from the group of pteric acid, neopterin,
5 pterin (3) tautomer and guanine (4) tautomer.

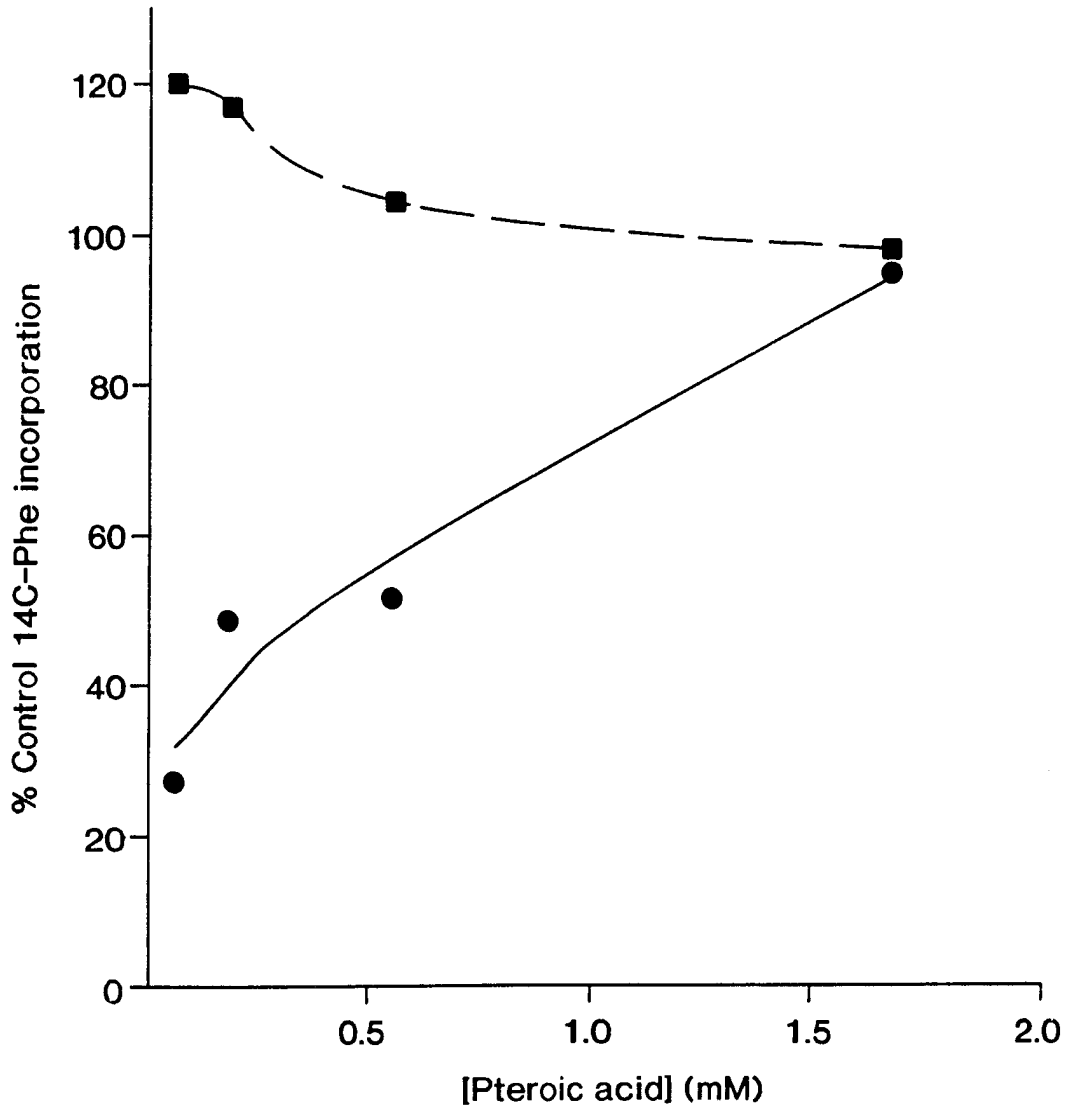


FIG. IA

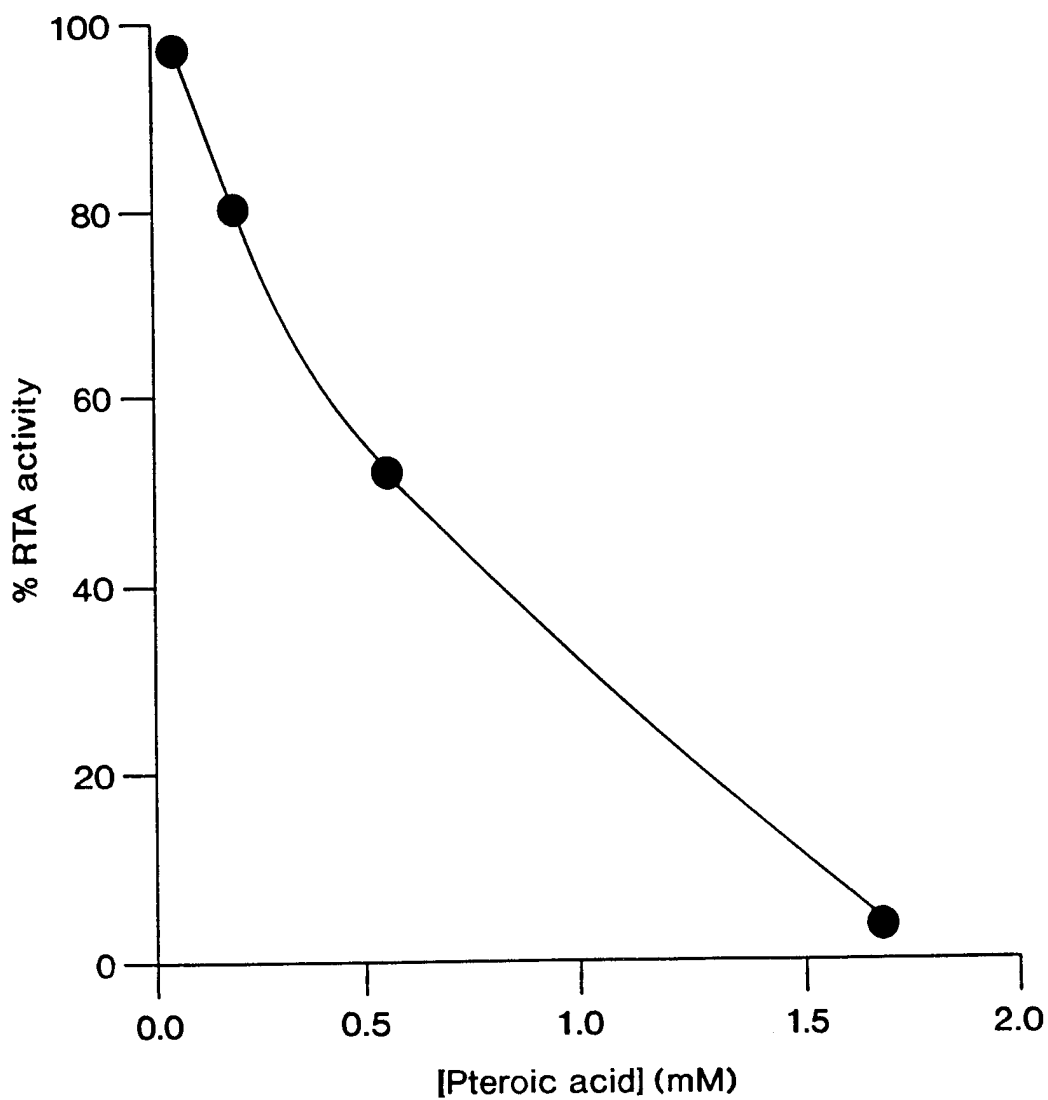


FIG. IB

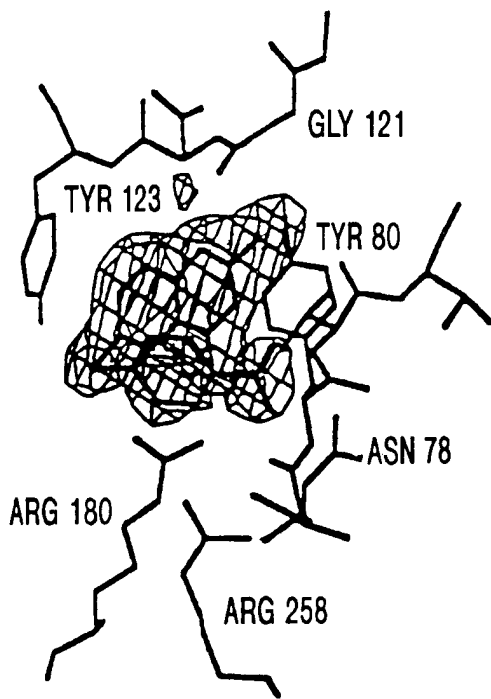


FIG. 2A

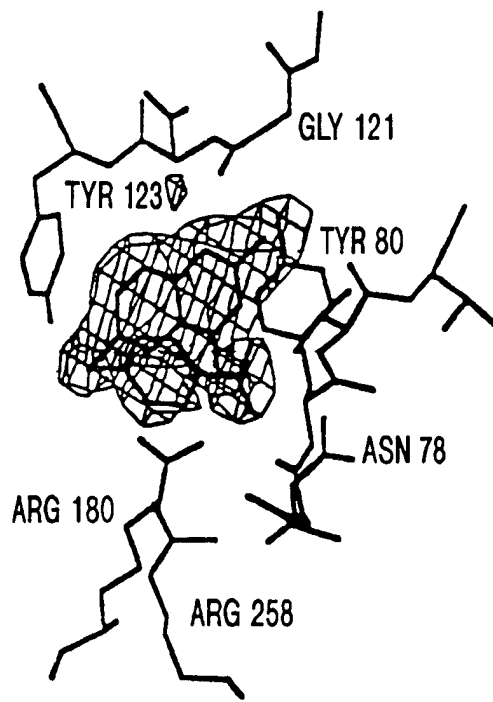


FIG. 2B

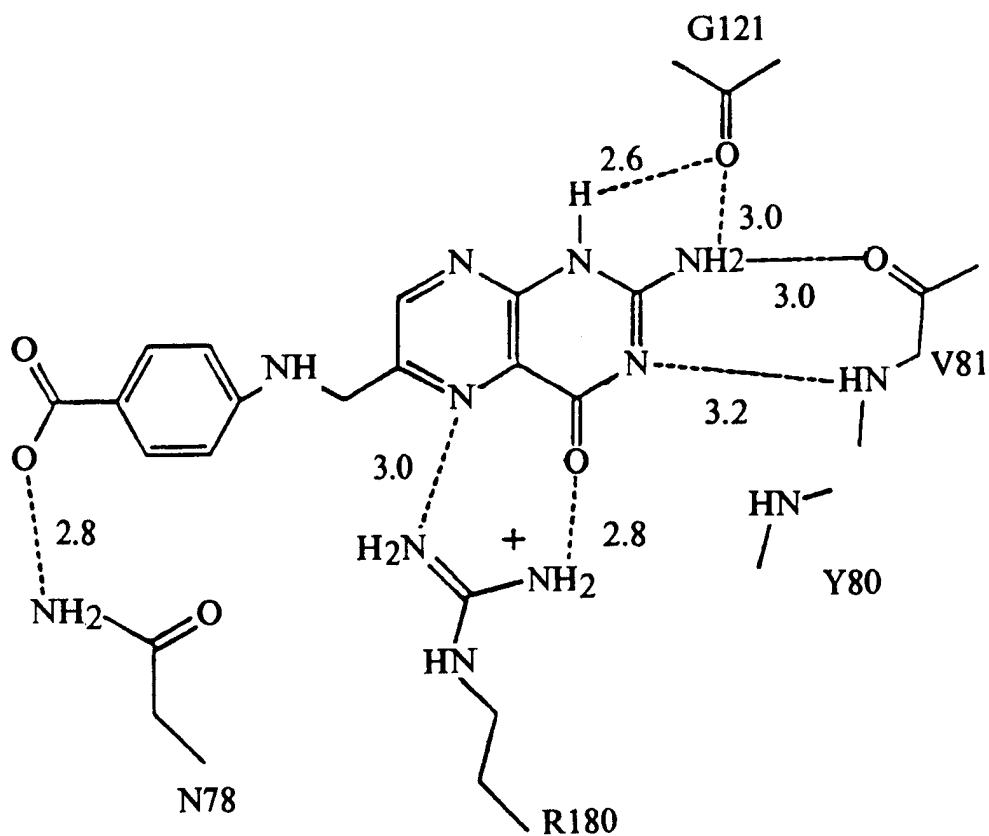


FIG. 3

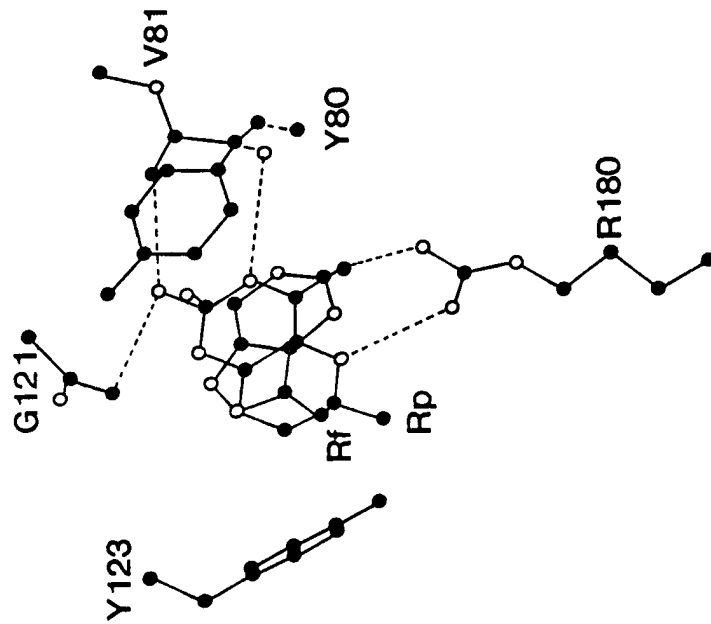


FIG. 4B

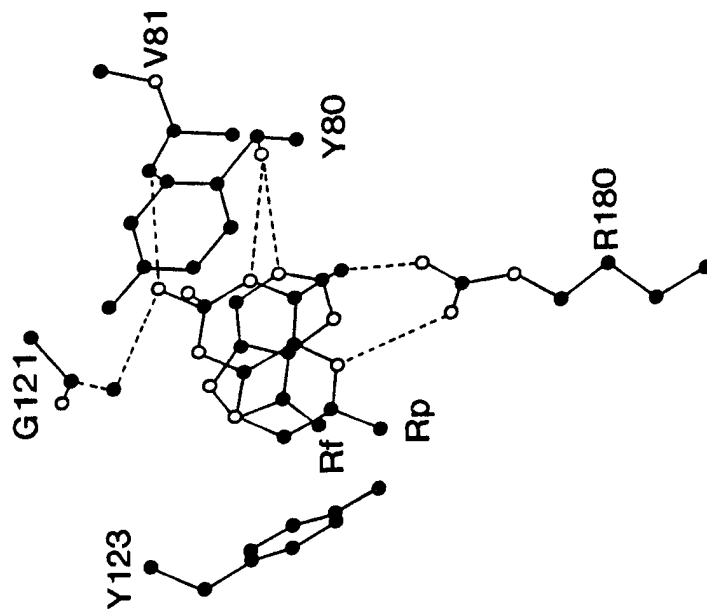


FIG. 4A

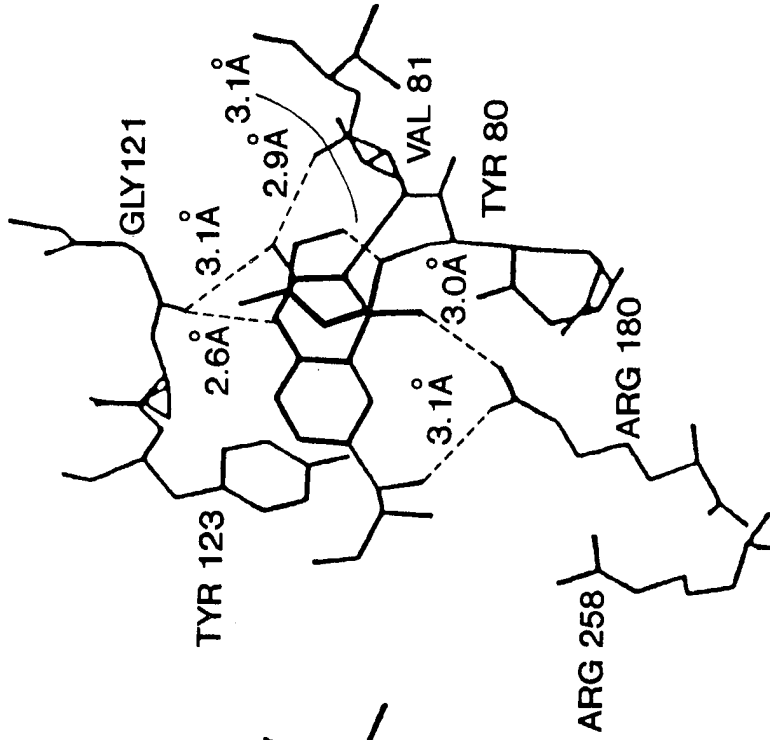


FIG. 5B

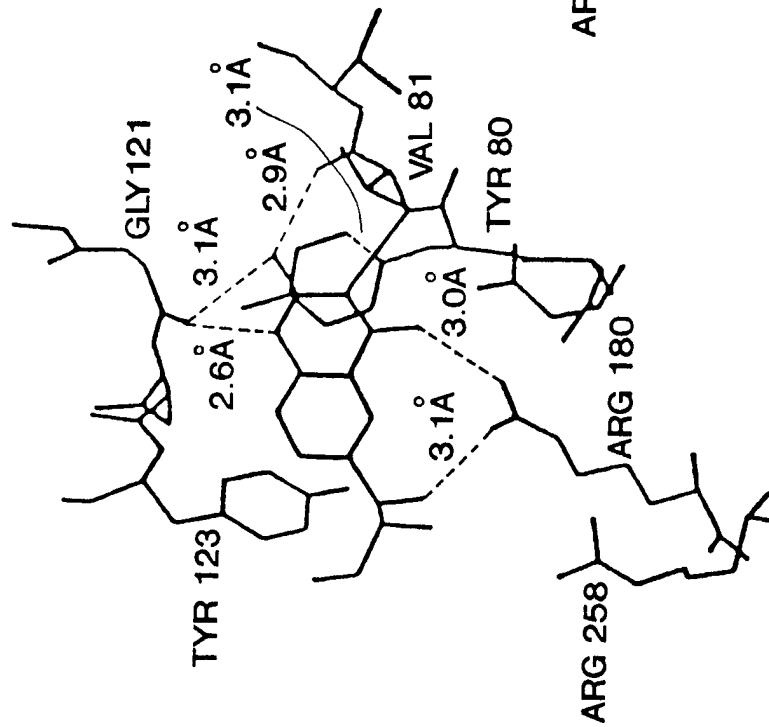
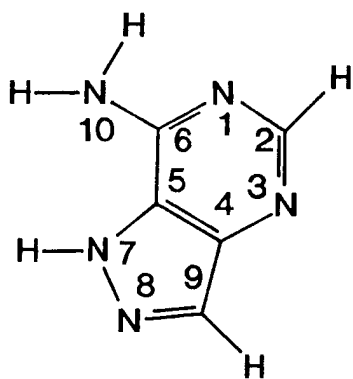
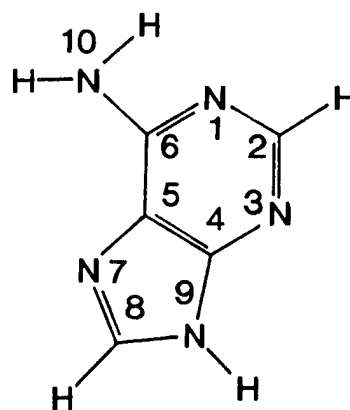


FIG. 5A



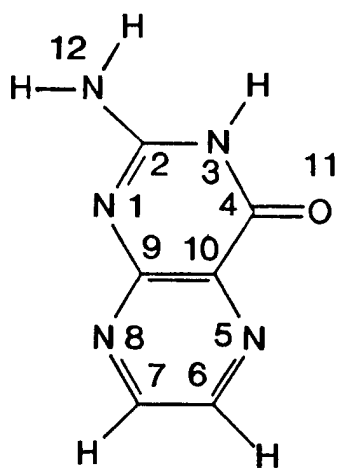
formycin

FIG. 6A



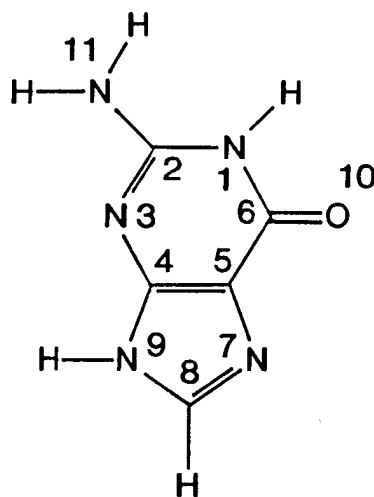
adenine(1)

FIG. 6B



pterin(1)

FIG. 6C



guanine(1)

FIG. 6D

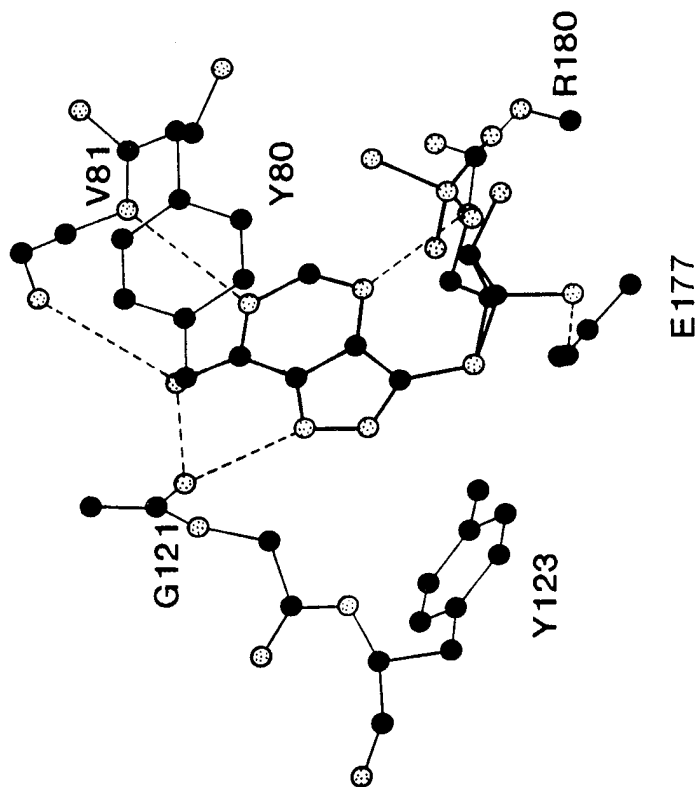


FIG. 7B

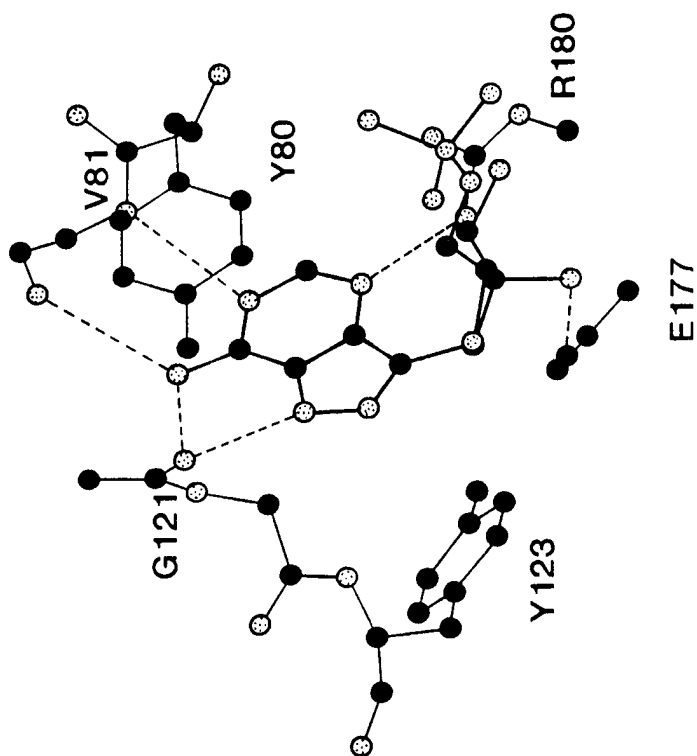
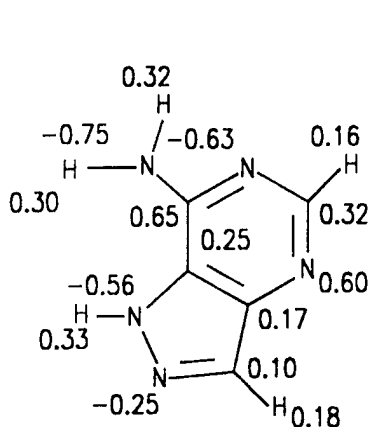
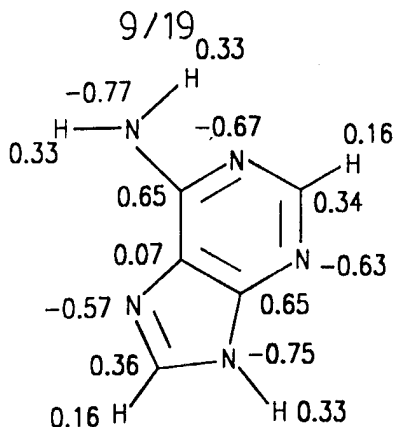


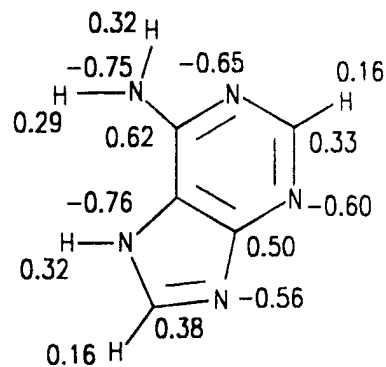
FIG. 7A



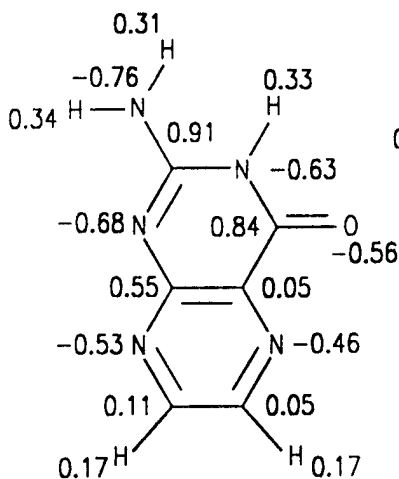
FORMYCIN
FIG. 8A



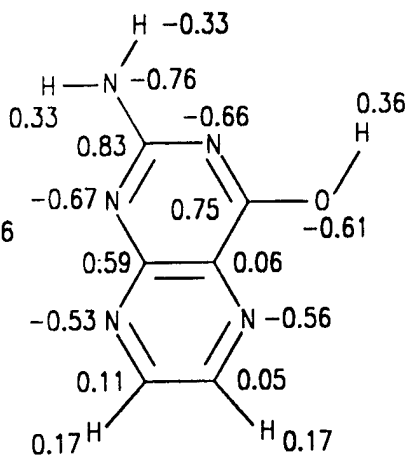
ADENINE(1)
FIG. 8B



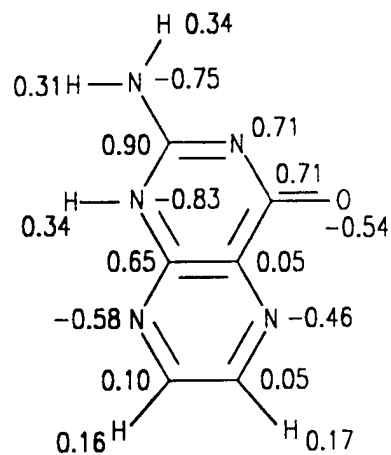
ADENINE(2)
FIG. 8C



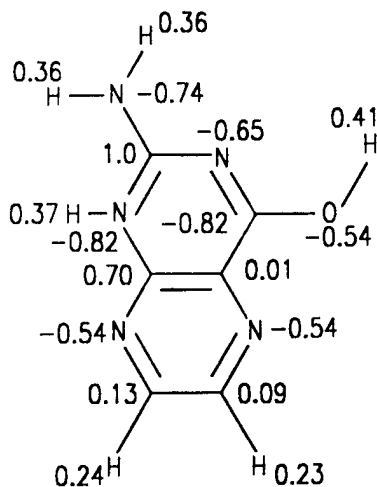
PTERIN(1)
FIG. 8D



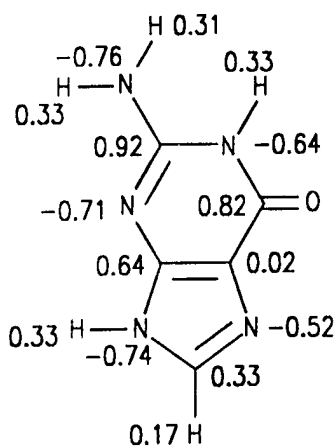
PTERIN(2)
FIG. 8E



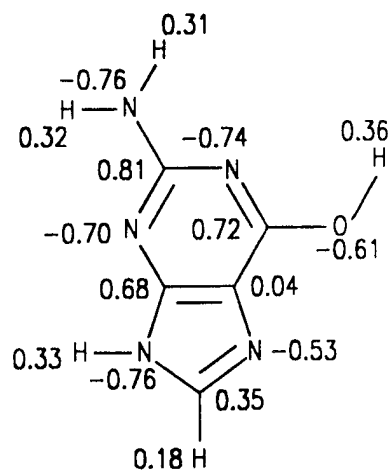
PTERIN(3)
FIG. 8F



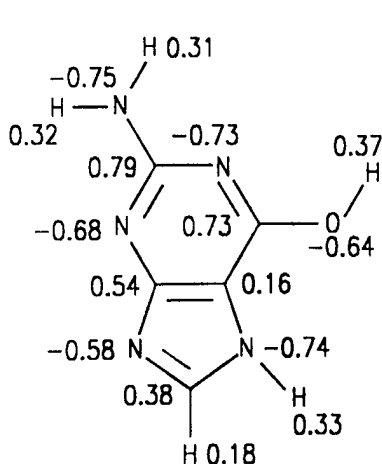
PTERIN(4 ION)
FIG. 8G



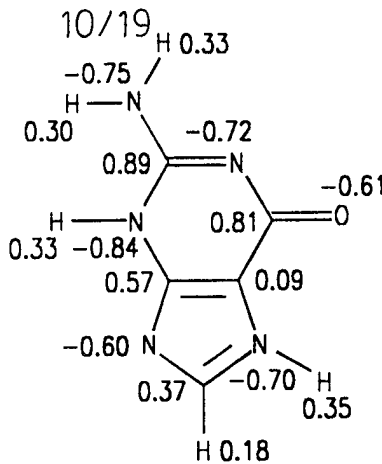
GUANINE(1)
FIG. 8H



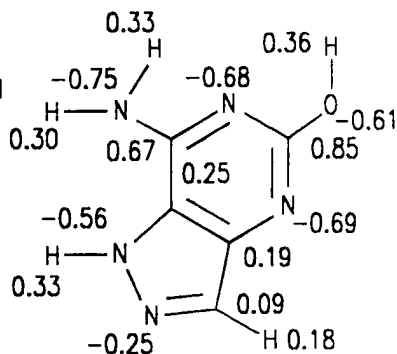
GUANINE(2)
FIG. 8I



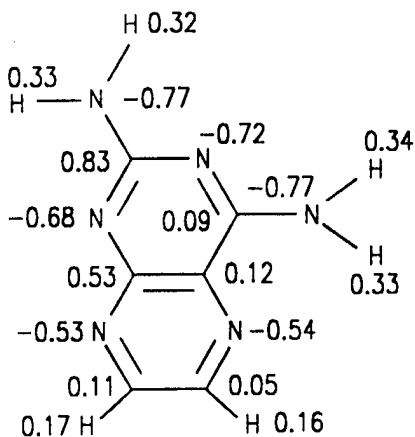
GUANINE(3)
FIG. 8J



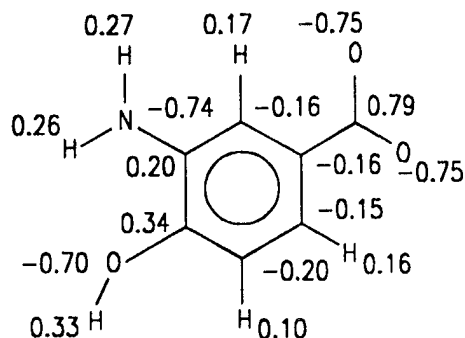
GUANINE(4)
FIG. 8K



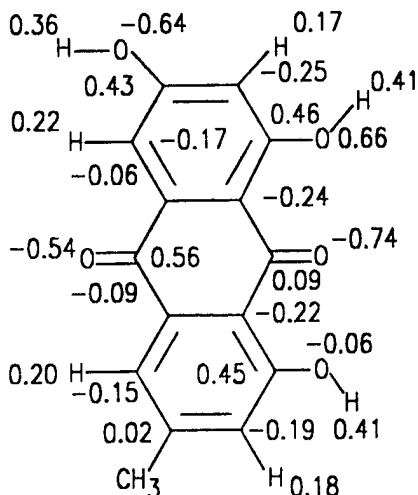
2-HYDROXY FORMYCIN
FIG. 8L



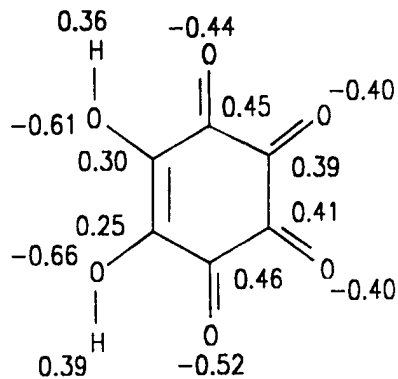
2,4-DIAMINO-PTERIDINE
FIG. 8M



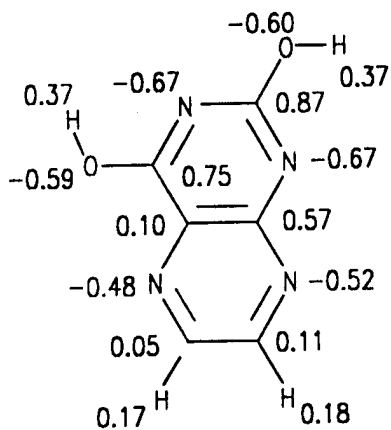
AHA(ION)
FIG. 8N



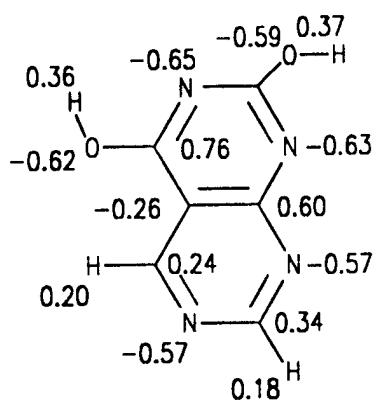
EMODINE
FIG. 8O



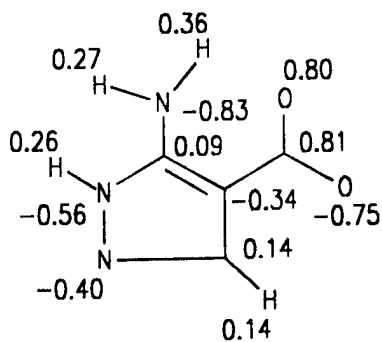
RHODIZONIC ACID
FIG. 8P



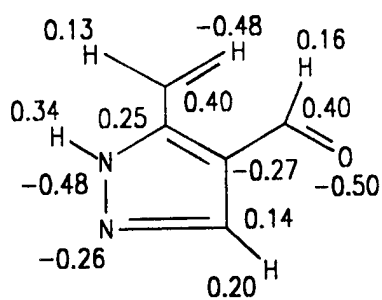
LUMAZINE
FIG. 8Q



S1
FIG. 8R



S2
FIG. 8S



S3
FIG. 8T

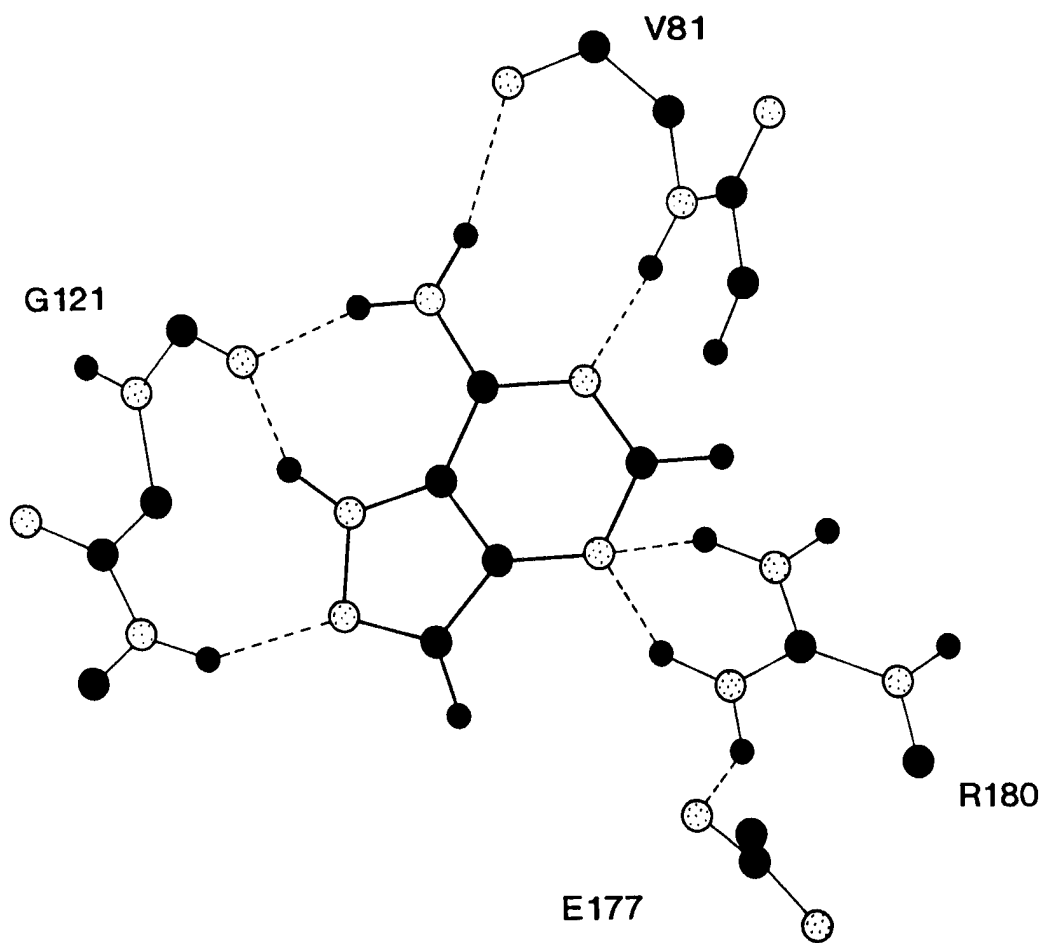


FIG. 9A

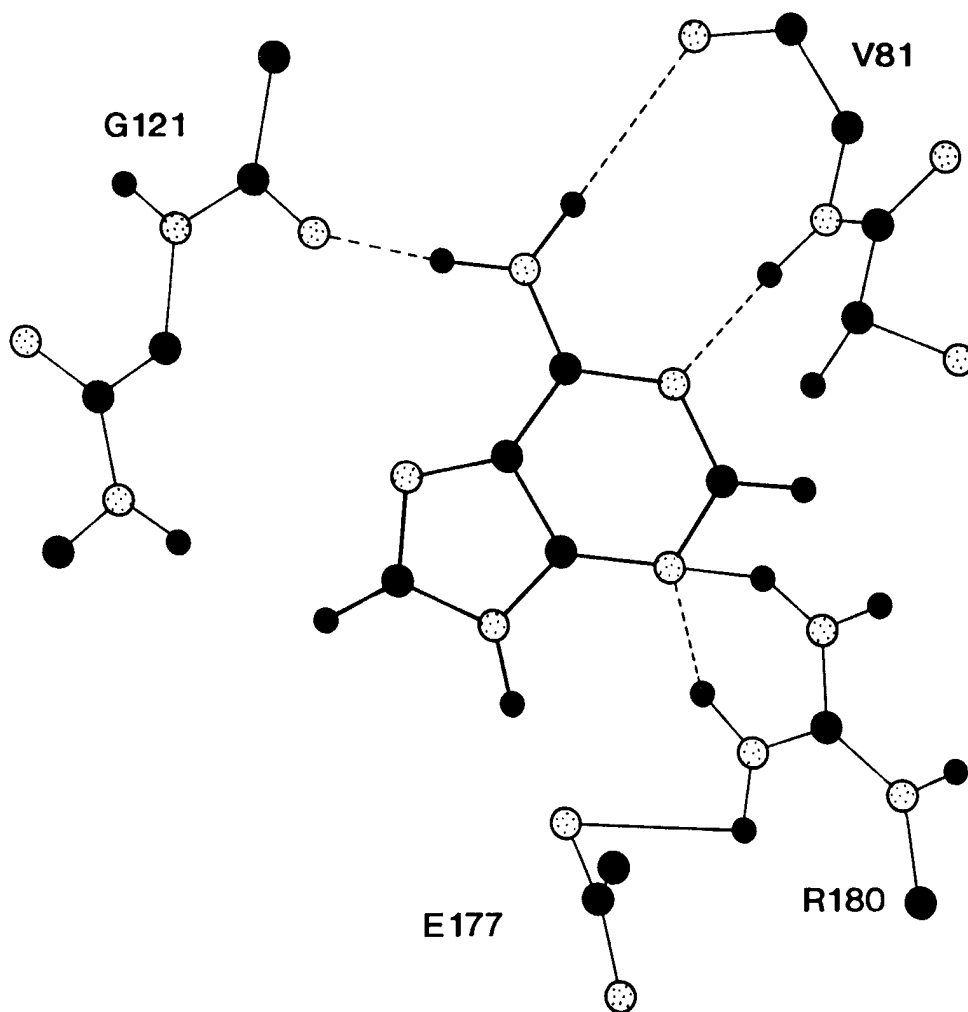


FIG. 9B

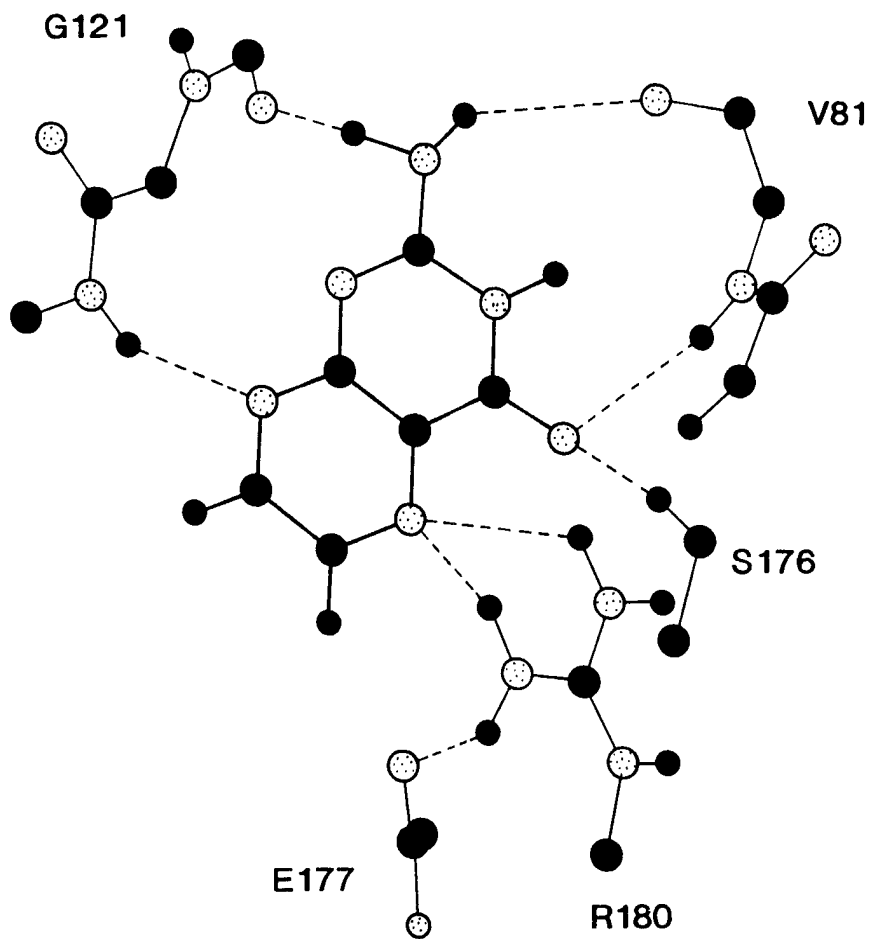


FIG. 9C

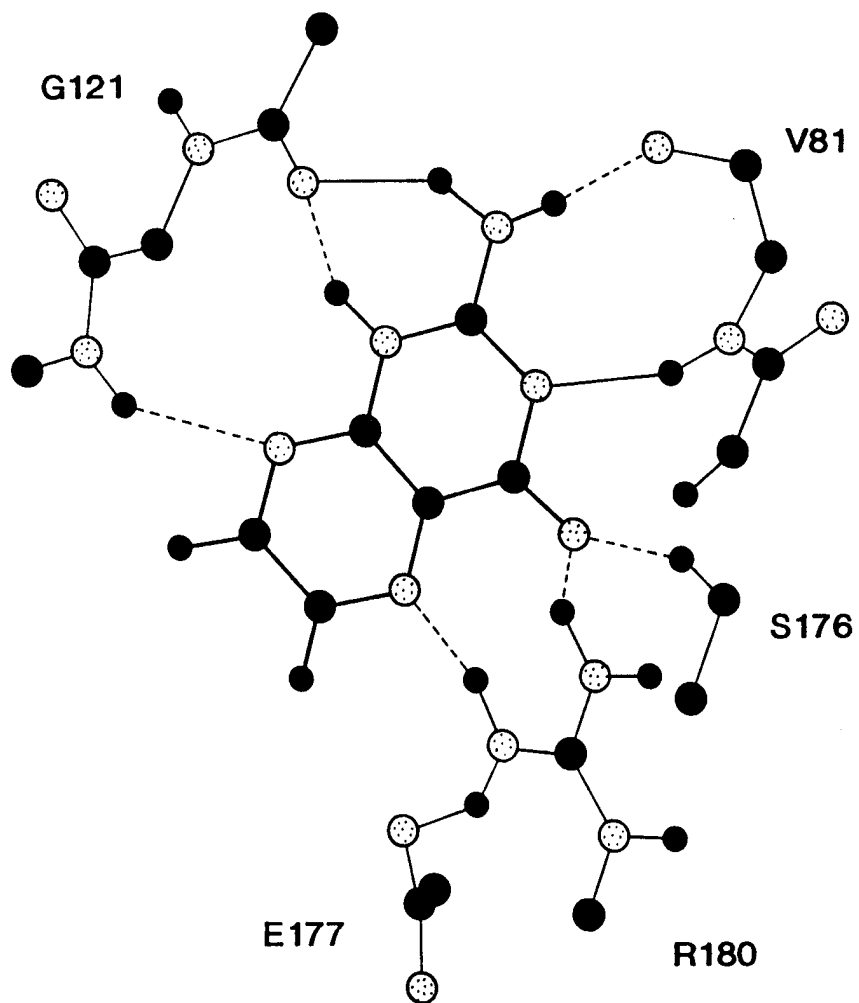


FIG. 9D

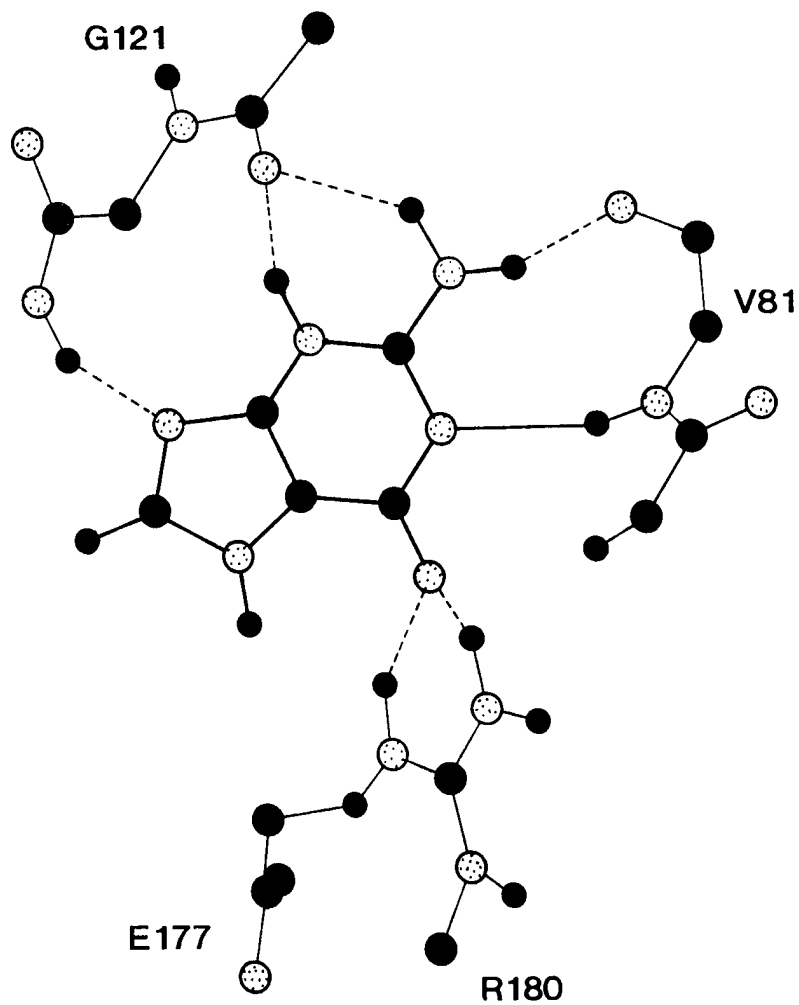


FIG. 9E

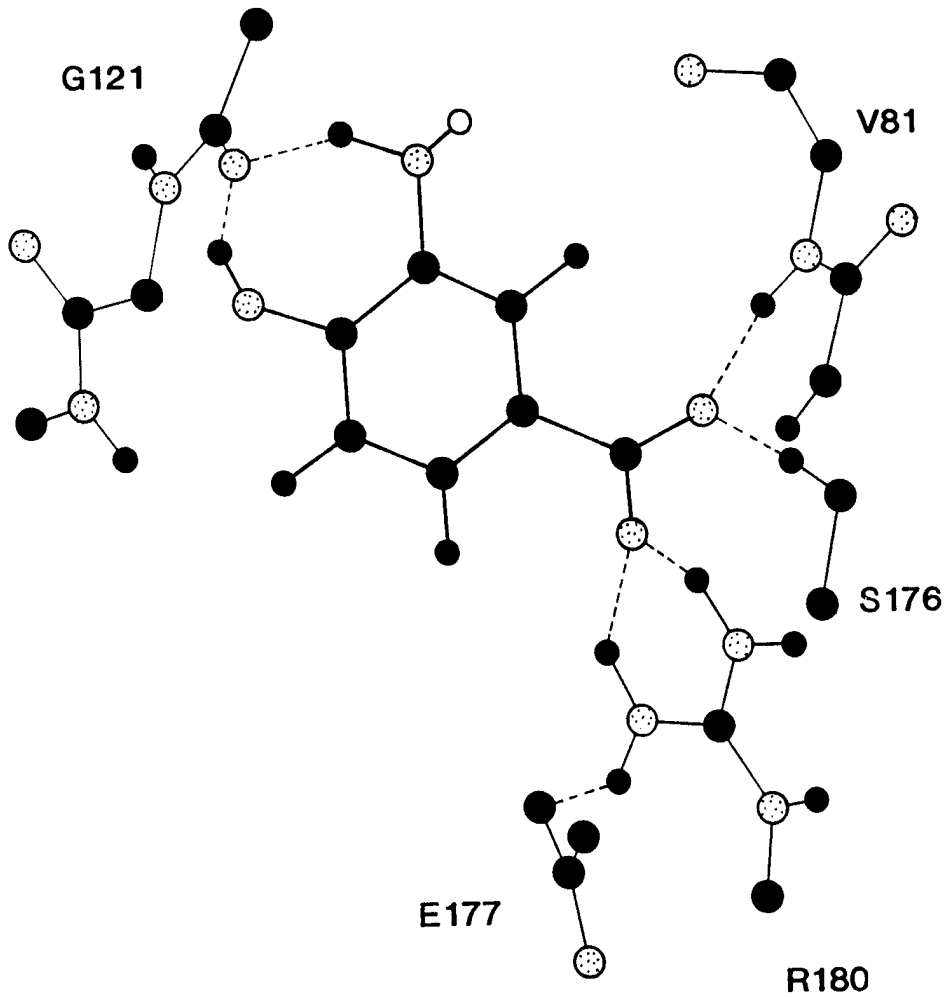


FIG. 9F

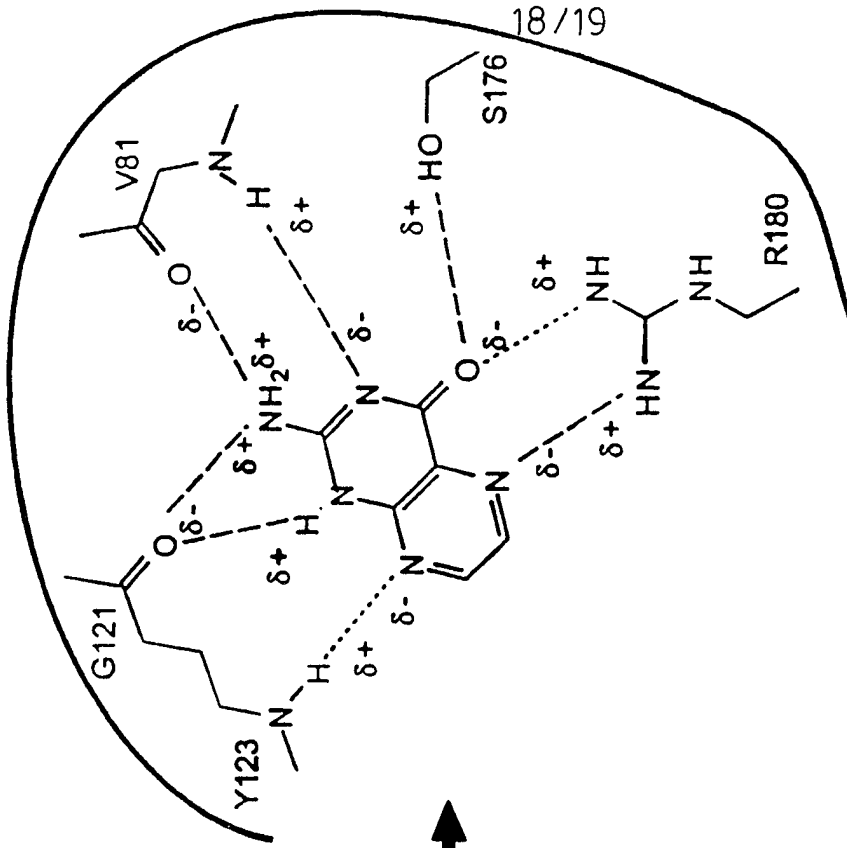


FIG. 10B

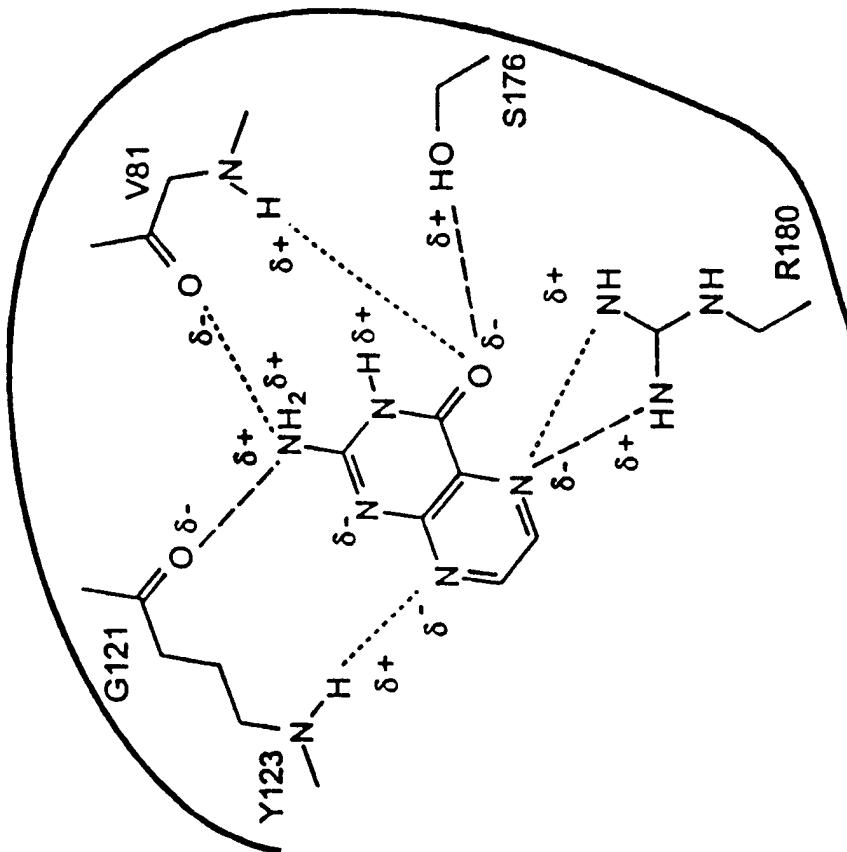


FIG. 10A

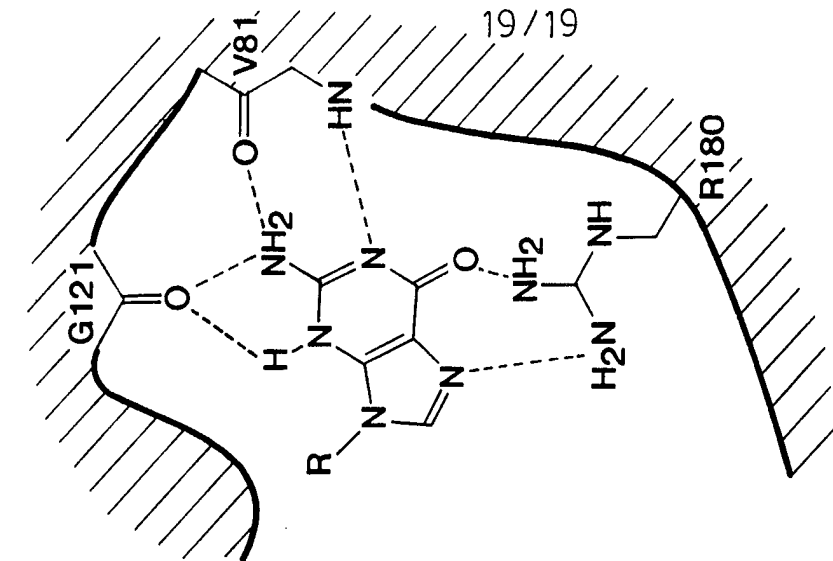


FIG. IIC

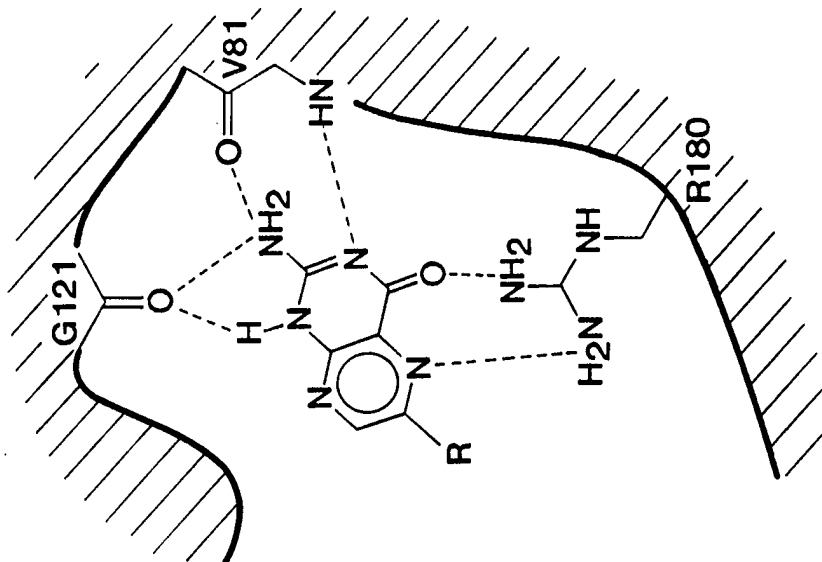


FIG. IIB

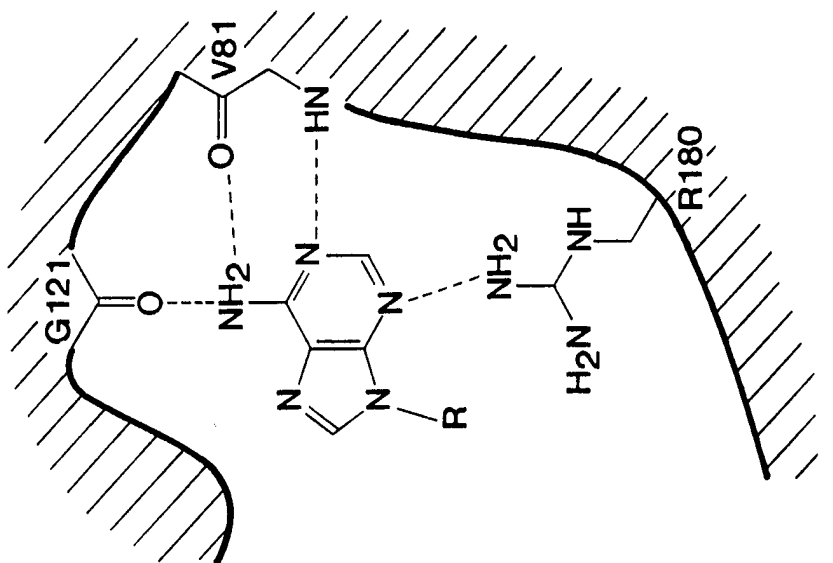


FIG. IIA

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/24144

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07D 475/00; G01N 33/53

US CL :544/251, 265; 436/501

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 544/251, 265; 436/501

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS ONLINE, APS

search terms: pteric acid, neopterin, pterin, guanine, ricin, inhibitor, Shiga toxin, RTA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2,472,462 A (BOOTHE) 07 June 1949, column 1, lines 5-9.	1-12
X	US 4,371,514 A (NAGATSU et al.) 01 February 1983, column 1, lines 8-11 and 31-35.	1-12
X	Stecher, P.G. (editor), The Merck Index, 1968, Merck & Co., Inc. publishers, page 510, "Guanine", see entire document.	1-12
A	US 4,340,535 A (VOISIN et al.) 20 July 1982, column 1, line 52 through column 2, line 2.	1-12

Further documents are listed in the continuation of Box C.

See patent family annex.

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