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(54) Title: HYDROLYTIC ENZYME-ACTIVATIBLE PRO-DRUGS

(57) Abstract

Antineoplastic agents are rendered tumor-specific by derivatization with a peptide specifier so as to convert the antineoplastic agent into a pharmacologically inactive pro-drug which is selectively activatible at the tumor site. The peptide specifier has an amino acid residue sequence such that it will be selectively enzymatically cleaved from the antineoplastic agent by tumor-associated fibrinolytic and/or blood-coagulating proteases, such as plasmin and plasminogen activator, so as to effect release of the antineoplastic agent in pharmacologically active form in the vicinity of the tumor. These and other similar hydrolytic enzyme-activatible pro-drugs may be formed with their specifier moiety and their drug moiety covalently linked together through an intermediate self-immolative connector moiety having a molecular structure such that enzymatic cleavage of the bond covalently linking it to the specifier moiety will initiate spontaneous cleavage of the bond covalently linking it to the drug moiety to thereby effect release of the drug in pharmacologically active form.

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Description

HYDROLYTIC ENZYME-ACTIVATIBLE PRO-DRUGS

BACKGROUND OF THE INVENTION

This invention relates to hydrolytic enzyme-activatible pro-drugs and, in particular, to tumor-specific pro-drugs of antineoplastic agents which are selective substrates for drug-activating enzymatic cleavage by tumor-associated proteases.

One approach to improving the efficiency of drug action and the selectivity of drug delivery is to prepare a reversible derivative of a drug which is itself pharmacologically inactive, but which becomes activated in vivo to liberate the parent drug, typically, but not necessarily, by enzymatic attack. A drug derivative of this type, commonly known as a "pro-drug" or a "latentiated drug", can be tailored to overcome certain undesirable properties of the parent drug, such as, for example, bitterness or tartness, offensive odor,gastric or intestinal upset and irritation, pain on injection, lack of absorption, slow or rapid metabolism, or lack of stability in the bulk state, the dosage form, or in vivo; or it can be designed to be activated selectively at the site of intended action, so that undesired effects can be lessened. The present invention is primarily concerned with pro-drugs of this latter type, which are selectively activatible at the site of intended action, and, in particular, to pro-drugs of antineoplastic agents which are selectively activatible at the tumor site.

One aspect of the present invention, however, is more broadly applicable to pro-drugs in general, as will become more readily apparent hereinbelow.

Many of the antineoplastic agents currently being used in cancer chemotherapy rely for their effectiveness on being selectively cytotoxic to rapidly proliferating cells. In addition to malignant cells, however, certain normal cells are also rapidly proliferating, such as, for example, bone marrow and spleen cells. For this reason bone marrow and spleen toxicity are often limiting factors in the effectiveness of such antineoplastic agents in cancer chemotherapy. One approach in trying to overcome this problem is to design a pro-drug of the antineoplastic agent which will be selectively activatible at the tumor-site, for example, by being a selective substrate for drug-activating enzymatic cleavage by a tumor-associated enzyme.

In order for a pro-drug of this type to be useful in cancer chemotherapy, there are several criteria which it must meet. First of all, there must be enough of the activating enzyme in the tumor to generate cytotoxic levels of free drug in the vicinity of the tumor. Secondly, there must be means available to minimize activation of the pro-drug at sites distant from the tumor, and to mitigate the effects of such activation if it occurs. This criterion is clearly related to the first one, since it is the relative amount of tumor-associated and extra-tumor enzymatic activity which is critical for selectivity. Thirdly, the pro-drug must be a suitable substrate for the tumor-associated enzyme under physiological conditions and a poor substrate for other enzymes. Fourthly, the pro-drug must be considerably less toxic than the activated drug, i.e., at least on the order of ten times less active and preferably on the order of a hundred or a thousand times less active. Finally, the

activated species must have a reasonably short biological half-life so that the toxic effects of the locally activated drug are limited to the tumor and selectivity is not lost by diffusion of the drug away from the site of activation.

A number of attempts have previously been made to develop tumor-specific pro-drugs of antineoplastic agents activatible by tumor-associated enzymes. However, such previous attempts have met with very limited success primarily due to a failure of such pro-drugs to meet one or more of the five criteria set forth above.

Another consideration in the design of hydrolytic enzyme-activatible pro-drugs, in general, is the problem sometimes posed by the nature of the drug molecule being derivatized. If the drug molecule is large or has pronounced polar or apolar character, steric or electronic factors at the intended cleavage site could interfere with the enzymatic cleavage reaction and thereby prevent the pro-drug from being a suitable substrate for the target enzyme.

It has previously been reported that many animal and human tumors exhibit elevated levels of fibrinolytic and blood-coagulating enzyme activity and, in particular, elevated levels of the fibrinolytic enzymes, plasmin and plasminogen activator. Both plasmin and plasminogen activator are proteases with trypsin-like specificity in the sense that they both cleave next to basic amino acids. Substantial information exists concerning the specificity of plasmin and plasminogen activator, based on the use of artificial substrates as well as analysis of the peptide bonds cleaved in the natural substrate. Plasminogen activator shows considerable substrate specificity towards its natural substrate, plasminogen, in which

a single Arg-Val bond is cleaved in converting plasminogen to plasmin. Plasmin is often regarded as a rather unspecific, trypsin-like protease. However, it cleaves a limited number of bonds in dissolving a fibrin clot. Examination of the plasmin cleavage sites in its natural substrate, fibrin, reveal that eleven of the fifteen earliest cleavages are at lysine residues, and in fifteen of the twenty earliest cleavages (including all of the earliest nine cleavages) a hydrophobic amino acid precedes the lysine or arginine. Hence, the implication is that plasmin is selective for lysine residues preceded by a hydrophobic amino acid residue.

The elevated levels of fibrinolytic and blood-coagulating enzymes found in many tumor cells and the substrate specificity of these proteases have not previously been exploited in the design of tumor-specific pro-drugs of antineoplastic agents. While it is true that various normal cells and tissues, including the lung, kidney, squamous epithelium and activated macrophages, also exhibit elevated levels of these proteases, such normal cells by and large are not rapidly proliferating, and thus should not be highly sensitive to the cytotoxic effects of a DNA synthesis inhibitor released in their vicinity. On the other hand, at least two major sites of high normal cell proliferation, i.e., the bone marrow and spleen, have been reported to be low in fibrinolytic and blood-coagulating enzyme activity. Hence, the specific combination of rapidly proliferating cells exhibiting high levels of fibrinolytic and blood-coagulating enzyme activity appears to be a characteristic possessed by a great many tumor cells but generally not possessed by normal cells.

SUMMARY OF THE INVENTION

It is, accordingly, a primary object of the present invention to provide a pro-drug of an antineoplastic agent which is selectively activatible at the site of the tumor.

Another object of the invention is to provide a tumor-specific pro-drug of an antineoplastic agent in accordance with the preceding object, which is a highly selective substrate for drug-activating enzymatic cleavage by one or more tumor-associated hydrolytic enzymes.

A further object of the invention is to provide a tumor-specific pro-drug of an antineoplastic agent in accordance with the preceding objects, wherein the activating enzyme is one which is present in the tumor in sufficient amounts to generate cytotoxic levels of free drug in the vicinity of the tumor.

Still another object of the invention is to provide a tumor-specific pro-drug of an antineoplastic agent in accordance with the preceding objects, wherein the activating enzyme is one whose presence at sites distant from the tumor is insufficient to generate cytotoxic levels of free drug in the vicinity of such distant sites.

A still further object of the present invention is to provide a tumor-specific pro-drug of an antineoplastic agent in accordance with the preceding objects, which is considerably less toxic than the activated drug.

Yet another object of the present invention is to provide a tumor-specific pro-drug of an antineoplastic agent in accordance with the preceding objects, wherein the activated drug has a reasonably short biological half-life so that the cytotoxic ef-

fects of the locally activated drug are limited to the tumor and selectivity is not lost by diffusion of the drug away from the site of activation.

A yet further object of the present invention is to provide hydrolytic enzyme-activatible pro-drugs, including those of the type set forth in the preceding objects, which include connector means for spacing the drug-activating enzymatic cleavage site sufficiently far away from the drug molecule so as to prevent steric and/or electronic interference with the enzymatic cleavage reaction, which connector means does not in itself prevent release of the free drug in pharmacologically active form following the enzymatic cleavage reaction.

The above and other objects are achieved in accordance with the present invention by derivatizing an antineoplastic agent with a peptide specifier at a reactive site appropriate for inhibiting the pharmacological activity of the antineoplastic agent, to thereby convert the antineoplastic agent into a pharmacologically inactive peptidyl derivative pro-drug. The peptide specifier has an amino acid residue sequence specifically tailored so as to render the peptidyl derivative a selective substrate for drug-activating enzymatic cleavage by one or more tumor-associated fibrinolytic and/or blood-coagulating proteases, such as plasmin and plasminogen activator. The enzymatic cleavage reaction will remove the peptide specifier moiety from the pro-drug and effect release of the antineoplastic agent in pharmacologically active form selectively at the tumor site.

In those instances where the drug molecule is large and/or has pronounced polar or apolar character, steric and/or electronic interference of the enzymatic cleavage reaction is avoided in accordance with the present

invention by forming the peptidyl derivative pro-drug with its peptide specifier moiety and its antineoplastic agent moiety covalently linked together through an intermediate self-immolative connector moiety having a molecular structure such that enzymatic cleavage of the bond covalently linking it to the peptide specifier moiety will initiate spontaneous cleavage of the bond covalently linking it to the antineoplastic agent moiety to thereby effect release of the antineoplastic agent in pharmacologically active form. The intermediate self-immolative connector aspect of the present invention is not limited in its application to protease-activatible pro-drugs of antineoplastic agents, but is equally applicable to a variety of other types of hydrolytic enzyme-activatible pro-drugs wherein steric and/or electronic hindrance by the drug molecule might otherwise interfere with the drug-activating enzymatic cleavage reaction. Moreover, the self-immolative connector aspect of the present invention may also be used to impart to the pro-drugs greater stability towards undesired hydrolytic processes, both enzymatic and spontaneous, and/or optimal pharmacokinetic properties without needing to chemically modify either the specifier or the drug themselves.

In vitro tests thus far carried out on several protease-activatible peptidyl derivative pro-drugs of antineoplastic agents in accordance with the present invention, show a five- to seven-fold improvement over the underivatized parent drug in selective cytotoxic activity against malignant cells exhibiting elevated levels of fibrinolytic enzyme activity versus well-matched (displaying similar good sensitivity to the free drug) normal cells not exhibiting such levels of fibrinolytic enzyme activity. These results are indicative of the fact that the peptidyl derivative pro-drugs of the present invention are selective substrates for drug-activating

enzymatic cleavage by tumor-associated fibrinolytic enzymes and are selectively activatible to release cytotoxic levels of pharmacologically active drug at sites exhibiting elevated levels of such fibrinolytic enzyme activity. Since normal tissues exhibiting such elevated levels of fibrinolytic enzyme activity are, for the most part, limited to those having a low percentage of replicating cells, peptidyl derivative pro-drugs of antineoplastic agents which are cytotoxic predominantly to rapidly proliferating cells in accordance with the present invention, should be selectively cytotoxic to those malignant cells which exhibit the specific combination of properties of being rapidly proliferating and exhibiting elevated levels of fibrinolytic enzyme activity.

DESCRIPTION OF PREFERRED EMBODIMENTS

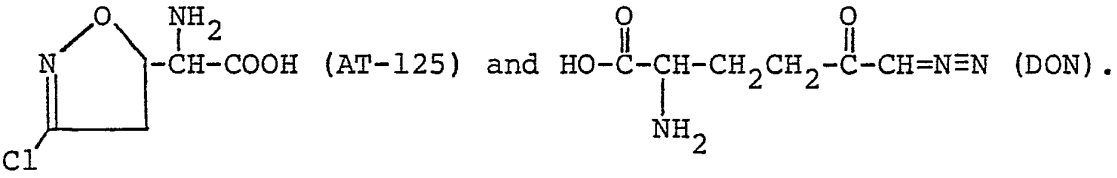
It will be understood that in the following detailed description and appended claims, the abbreviations and nomenclature employed are those which are standard in amino acid and peptide chemistry, and that all amino acids referred to are in the L-form unless otherwise specified.

The hydrolytic enzyme-activatible pro-drugs in accordance with the present invention may be broadly described as having a molecular structure comprised of a drug moiety and a specifier moiety. The specifier moiety, by means of its chemical structure, targets the pro-drug to one or more species of hydrolytic enzymes, and renders the pro-drug a selective substrate for drug-activating enzymatic cleavage by the target hydrolytic enzyme. The drug moiety and the specifier moiety are covalently linked together either directly to form a bipartate molecular structure, or through an intermediate self-immolative connector moiety to form a tripartate molecular structure. In either case, the covalent

linkage between the moieties will be such that the drug moiety is rendered pharmacologically inactive, the site of the drug-activating enzymatic cleavage will be at the bond covalently linking the specifier moiety to its immediately adjacent moiety, and the drug-activating enzymatic cleavage will effect release of the drug moiety in pharmacologically active form. The intermediate self-immolative connector moiety, when employed in the pro-drug molecule, has a molecular structure such that the drug-activating enzymatic cleavage of the bond covalently linking it to the specifier moiety will initiate spontaneous cleavage of the bond covalently linking it to the drug moiety, to thereby effect release of the drug moiety in pharmacologically active form.

The peptidyl derivative pro-drugs of antineoplastic agents in accordance with the present invention have an antineoplastic agent as their drug moiety and a peptide as their specifier moiety,and are specifically designed to be selective substrates for drug-activating enzymatic cleavage by one or more tumor-associated proteases selected from the group consisting of fibrinolytic enzymes and blood-coagulating enzymes. Blood-coagulating enzymes are those which are involved in the intrinsic or extrinsic system of fibrin clot formation, and include, but are not necessarily limited to, thrombin, thromboplastin, Factor Va, Factor VIIa, Factor VIIIa, Factor IXa, Factor Xa, Factor XIa, and Factor XIIa. Fibrinolytic enzymes are those which are involved in the physiological mechanism for dissolving fibrin clots, and include plasmin and plasminogen activator. Recent evidence suggests that all of these proteases are associated with a great many tumors, and that plasmin and plasminogen activator, in particular,are present in these tumors at elevated levels sufficient for pro-drug activation.

In order to be suitable for conversion into a pro-drug in accordance with the present invention, the anti-neoplastic agent should be one having an unhindered chemically reactive site whose derivatization will inhibit the pharmacological activity of the antineoplastic agent. Such reactive site will typically be a free amino group or a free hydroxyl group, since these groups are most readily derivatizable with peptides. However, where an intermediate self-immolative connector is employed in forming the pro-drug, the reactive site for derivatization of the antineoplastic agent may also be a free sulfhydryl group. A number of known antineoplastic agents meet the above requirements, including, for example, cytosine arabinoside, adriamycin, daunomycin, 6-thioguanine, fluorodeoxyuridine, bis-(2-chloroethyl) amine, phenylenediamine mustard, 3'-aminothymidine, L-alanosine, 2-aminothiodiazole, 1,4-dihydroxy-5,8-bis(2-aminoethylamino)-9,10-anthracenedione,



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The peptide specifier employed for derivatizing the antineoplastic agent so as to convert it into a tumor-specific pro-drug in accordance with the present invention, has an amino acid residue sequence specifically tailored so that it will be selectively enzymatically cleaved from the resulting peptidyl derivative pro-drug by one or more of the tumor-associated fibrinolytic and/or blood-coagulating proteases. Examination of the cleavage sites in the natural substrates for these proteases provides a basis for choosing appropriate amino acid residue sequences for the peptide specifier. Since at least most of the fibrinolytic and blood-coagulating proteases appear to have in common a relatively high degree of specificity toward cleavage sites in their natural substrates which have a

basic amino acid residue on the carboxyl side thereof, it is preferred to form the peptide specifier with a basic amino acid residue in its C-terminal position, and to carry out the derivatization of the antineoplastic agent with the C-terminus of the peptide specifier. Suitable basic amino acid residues for use as the C-terminal amino acid residue of the peptide specifier include lysine, arginine, histidine, ornithine, and citrulline, with lysine and arginine being particularly preferred.

The amino acid residue in the position immediately adjacent to the C-terminal amino acid residue also appears to play a significant role in imparting the desired protease-specificity to the peptide specifier. Such penultimate amino acid residue is preferably a hydrophobic amino acid residue or glycine. Suitable hydrophobic amino acid residues include alanine, valine, leucine, isoleucine, methionine, phenylalanine, tryptophan and proline. Alanine, leucine and glycine are particularly preferred amino acid residues for use in such penultimate position of the peptide specifier.

For facilitating preparation of the pro-drug and enhancing its stability against undesired hydrolytic processes, the N-terminal amino acid residue of the peptide specifier is preferably a D-amino acid residue, a protected L-amino acid residue, or protected glycine. Suitable protecting groups are well known in the art of peptide chemistry, and include,for example carbobenzoxy (CBZ), t-butoxycarbonyl (Boc), p-toluene sulfonyl, and benzoyl, with CBZ being particularly preferred. Most preferably, the N-terminal amino acid residue is a D-amino acid residue, such as, for example, D-valine or D-isoleucine, since this provides the peptide specifier with better solubility properties than with the protected L-amino acid residue or protected glycine.

The amino acid residue chain length of the peptide specifier preferably ranges from that of a tripeptide to that of a pentadecapeptide. It will be understood, however, that peptide specifiers as short as dipeptides and longer than pentadecapeptides may also suitably be employed.

With the foregoing basic considerations serving as a general guideline, numerous specific peptide specifier molecules suitable for use in the present invention can be designed and optimized in their selectivity for enzymatic cleavage by a particular one of the tumor-associated fibrinolytic and blood-coagulating proteases. Based upon the information which is presently available in regard to the cleavage site specificities and the tumor-associated concentrations of these proteases, the presently preferred peptide specifiers for use in the present invention are those which are optimized toward the fibrinolytic proteases, plasmin and plasminogen activator. Its high degree of cleavage site specificity makes plasminogen activator a particularly attractive target protease from the standpoint of designing pro-drugs with optimal selectivity. On the other hand, since one plasminogen activator molecule is capable of converting numerous molecules of plasminogen to plasmin, plasmin will generally have a substantially greater tumor-associated concentration than plasminogen activator and, notwithstanding its lower degree of cleavage site specificity, may be more likely to provide a target large enough to generate pharmacologically significant concentrations of the antineoplastic agent from the pro-drug. In any event, both plasminogen activator, due to its high degree of cleavage site specificity, and plasmin, due to its high tumor-associated concentration, appear to be the target proteases of choice in determining optimal amino acid residue sequences for the

peptide specifier under the aforementioned general guidelines.

In the peptide specifiers optimized toward plasmin as the target protease, the C-terminal amino acid residue is preferably lysine, the amino acid residue in the position immediately adjacent to the C-terminal amino acid residue is preferably leucine, and the N-terminal amino acid residue is preferably D-valine or D-isoleucine. Specific examples of this preferred embodiment of peptide specifiers include the tripeptides D-Val-Leu-Lys and D-Ile-Leu-Lys, and the tetrapeptides D-Val-Ser-Leu-Lys and D-Ile-Ser-Leu-Lys.

In the peptide specifiers optimized toward plasminogen activator as the target protease, the amino acid residue sequence preferably substantially mimics the amino acid residue sequence on the carboxyl side of the Arg-Val bond in plasminogen which serves as the site of cleavage of plasminogen by plasminogen activator, with the C-terminal amino acid residue preferably being arginine, and the amino acid residue in the position immediately adjacent to the C-terminal amino acid residue being glycine. Specific examples of this preferred embodiment of peptide specifiers include the tripeptide CBZ-Pro-Gly-Arg, the tetrapeptide CBZ-Cys(R)-Pro-Gly-Arg, the pentapeptide CBZ-Lys-Cys(R)-Pro-Gly-Arg, and the hexapeptide CBZ-Lys-Lys-Cys(R)-Pro-Gly-Arg. In addition, CBZ-Gly-Gly-Arg is a suitable preferred specifier.

Optimization of the peptide specifier toward one or more of the blood-coagulating enzymes as the target tumor-associated protease may similarly be accomplished by choosing an amino acid residue sequence in accordance with the aforementioned general guidelines, but which substantially mimics the amino acid residue sequence on the carboxyl side of the cleavage site in the appropriate natural or known artificial substrates for the particular enzyme. Examples of such substrates are disclosed by Claeson, et al, "Substrate Structure and Activation Relationship", appearing in New Methods For the Analysis of Coagulation Using Chromogenic Substrates, Ed. I. Witt, Walter de Gruyter, Berlin, New York, Pages 37-54 (1977), incorporated herein by reference. Representative peptide specifiers within the scope of the present invention and optimized toward thrombin as the target protease, include the tripeptides p-toluene sulfonyl-Gly-Pro-Arg and benzoyl-Phe-Val-Arg. A representative peptide specifier in accordance with the present invention and optimized toward Factor Xa as the target protease is the tetrapeptide benzoyl-Ile-Glu-Gly-Arg.

In the preferred procedure for synthesizing the peptidyl derivative pro-drugs in accordance with the present invention, the peptide specifier is first separately prepared with its C-terminus in the free acid form, and with all of its other reactive groups suitably blocked. Synthesis of the peptide specifier may be carried out by standard peptide synthesis techniques well known in the art, including either solution-phase or solid-phase methods. Particularly where the peptide being synthesized is of relatively short chain length, the solution-phase methods offer certain advantages in that the peptide is directly prepared in the blocked form needed for the subsequent derivatization of the drug, and the intermediates in the synthesis can be purified,

insuring product peptide purity. If solid-phase methods are employed, various known techniques may be used for the removal of the blocked peptide from the resin, for example, by using either photocleavable attachment linkages, or by acyl transfer with 2-dimethylaminoethanol followed by hydrolysis.

If the antineoplastic agent being converted into a pro-drug contains more than one reactive site on its molecule, those reactive sites other than the one being derivatized may be suitably protected prior to the derivatization reaction. Any of the conventional protecting groups well known in the art may suitably be used for this purpose. For example, in derivatizing the 5'-hydroxyl group of cytosine arabinoside, the 4-amino group of the base may suitably be protected as the Schiffs base using dimethylformamide dimethyl ketal or diisopropylformamide dimethyl ketal, with the latter providing more favorable organic solubility characteristics important in attaining good product recoveries. Deprotection following the derivatization reaction may be effected with trifluoroacetic acid. Another instance where protecting groups might be used to achieve more favorable organic solubility characteristics would be in the derivatization of the 2-amino group of 6-thioguanine, wherein benzylation of the 6-thio group and at the N^4 position will increase the solubility of the parent drug. Deprotection of S and N benzyl protecting groups following the derivatization reaction can be accomplished by treatment with sodium in liquid ammonia.

In preparing the bipartate peptidyl derivative pro-drugs in accordance with the present invention, the peptide specifier, with its C-terminus in the free acid form, and with all of its other reactive groups suitably blocked, is directly reacted with a carboxyl-reactive site of the antineoplastic agent whose

derivatization inhibits pharmacological activity, to thereby form a direct covalent linkage between the C-terminus of the peptide specifier and said carboxyl-reactive site of the antineoplastic agent. Such covalent linkage will either be an amide linkage, i.e., when the carboxyl-reactive site is a free amino group; or an ester linkage, i.e., when the carboxyl-reactive site is a free hydroxyl group. Where a choice is available between synthesizing the pro-drug with either an amide linkage or an ester linkage, amide linkages are generally preferred in view of the fact that ester-linked pro-drugs tend to lose at least some selectivity due to hydrolysis by non-specific esterases.

Standard ester-forming and amide-forming techniques well known in the art may be used for carrying out the derivatization reaction. For example, the amide-linked derivatives may suitably be prepared via a mixed anhydride reaction with the aid of isobutyl chloroformate and either triethylamine or N-methyl morpholine in a suitable solvent such as dimethylformamide or dioxane/tetrahydrofuran. Following the derivatization reaction, the protecting groups are removed, for example, by treatment with trifluoroacetic acid in methylene chloride, to yield the desired peptidyl derivative pro-drug.

The tripartate pro-drugs in accordance with the present invention employ an intermediate self-immolative connector moiety which spaces and covalently links together the drug moiety and the specifier moiety. Since the self-immolative connector aspect of the present invention is believed to be a novel concept in the design of hydrolytic enzyme-activatible pro-drugs in general, it will be described, first of all, in terms of its broader applications, and thereafter,

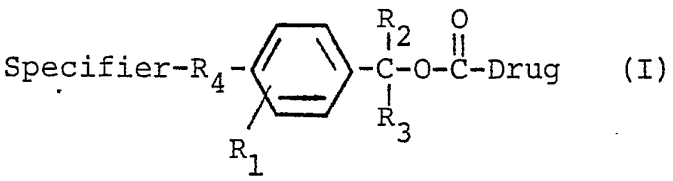
as it more specifically relates to the peptidyl derivative pro-drugs of antineoplastic agents in accordance with the present invention.

In its broadest sense, a self-immolative connector may be defined as a bifunctional chemical moiety which is capable of (1) covalently linking together two spaced chemical moieties into a normally stable tripartate molecule; (2) releasing one of said spaced chemical moieties from the tripartate molecule by means of an enzymatic cleavage; and (3) following said enzymatic cleavage, spontaneously (i.e., non-enzymatically) cleaving from the remainder of the molecule to release the other of said spaced chemical moieties. As applied to the design of hydrolytic enzyme-activatible pro-drugs in accordance with the present invention, the self-immolative connector is covalently linked at one of its ends to the specifier moiety and covalently linked at its other end to the reactive site of the drug moiety whose derivatization inhibits pharmacological activity, so as to space and covalently link together the specifier moiety and the drug moiety into a tripartate molecule which is stable and pharmacologically inactive in the absence of the target hydrolytic enzyme, but which is enzymatically cleavable by such target hydrolytic enzyme at the bond covalently linking the connector moiety to the specifier moiety to thereby effect release of the specifier moiety from the tripartate molecule. Such enzymatic cleavage, in turn, will activate the self-immolating character of the connector moiety and initiate spontaneous cleavage of the bond covalently linking the connector moiety to the drug moiety, to thereby effect release of the drug in pharmacologically active form.

A self-immolative connector offers several potential advantages in hydrolytic enzyme-activatible pro-drug design. First of all, in those instances where the drug molecule being derivatized is large and/or has pronounced polar or apolar character, a bipartate pro-drug formed by a direct linkage of the specifier moiety to the drug moiety may not be a suitable substrate for the target hydrolytic enzyme due to steric and/or electronic hindrance at the intended enzymatic cleavage site caused by the close proximity of such site to the drug molecule. By inserting an intermediate self-immolative connector moiety between the specifier moiety and the drug moiety, the drug-activating enzymatic cleavage site may be spaced sufficiently far away from the drug molecule so as to prevent steric and/or electronic interference with the enzymatic cleavage reaction, and without the connector itself preventing release of the free drug in pharmacologically active form following the enzymatic cleavage reaction. This should allow construction of many more classes and types of hydrolytic enzyme-activatible pro-drugs. Secondly, by varying the functionality of the drug-derivatizing end of the self-immolative connector from that of the specifier, the self-immolative connector may provide greater versatility in the type of linkage used for derivatizing the drug, and may enable linkages which are more stable towards undesired hydrolytic processes (both enzymatic and spontaneous) than are the direct specifier-drug linkages. Thirdly, by providing the self-immolative connector with numerous sites for chemical substitution, it should be possible to design tripartate pro-drugs with optimal pharmacokinetic properties without needing to chemically modify either the specifier or the drug

themselves. This should allow the specifier and drug to be individually optimized for their own particular tasks, e.g., the specifier might be optimized as a substrate for the desired hydrolytic enzyme and made relatively resistant to hydrolysis by undesired hydrolytic enzymes, and the drug might be optimized for specific inhibition of some target enzyme.

A connector moiety which has been found to have all of the above-described characteristics rendering it particularly suitable for use as a self-immolative connector, and the manner in which it is employed in the design of hydrolytic enzyme-activatible tripartate pro-drugs in accordance with the present invention, may be represented by the following general formula:



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wherein R_1 is hydrogen or one or more substituent groups which are either electron-donating groups or electron-withdrawing groups; R_2 and R_3 may be the same or different and are each selected from the group consisting of hydrogen, alkyl, phenyl, and phenyl substituted with either electron-donating groups or electron-withdrawing groups; R_4 is NH or O; when R_4 is NH, the specifier moiety is selected from the group consisting of a peptide, an amino acid, a carboxylic acid, and phosphoric acid; when R_4 is O, the specifier moiety is selected from the group consisting of a carboxylic acid, phosphoric acid, and sulfuric acid; and the drug moiety is a normally pharmacologically active agent having a reactive site whose derivatization inhibits pharmacological activity, said reactive site being selected from the group consisting of a

free amino group, a free hydroxyl group and a free sulfhydryl group, the covalent linkage between said drug moiety and its adjacent carbonyl group being at said reactive site so as to inhibit the pharmacological activity of said drug moiety.

In the above definitions of R_1, R_2 and R_3, any of the common electron-donating and electron-withdrawing groups well known in the art may suitably be employed. By way of example suitable electron-donating groups include $-NH_2$, $-OH$, $-OCH_3$, $-NHCOCH_3$, $-C_6H_5$, and $-CH_3$; and suitable electron-withdrawing groups include $-NH^+(CH_3)_3$, $-NO_2$, $-CN$, $-SO_3H$, $-COOH$, $-CHO$, $-COR$, $-Cl$, $-Br$, $-I$, and $-F$. In the above definitions of R_2 and R_3, the alkyl group will generally be a lower alkyl group but may, if desired, be of longer chain length, for example, up to about 15 carbon atoms. Preferred embodiments of the connector moiety are those in which R_1 is hydrogen, R_2 is hydrogen or methyl, and R_3 is hydrogen or methyl.

In Formula I, above, the specifier moiety and the R_4 group together constitute a substrate recognition site for a particular class of target hydrolytic enzymes, which is dependent upon the specific combination of specifier moiety and R_4 group selected. The various classes of target hydrolytic enzymes for each specific combination of specifier moiety and R_4 group within the Formula I definitions set forth above, are listed in Table I, below.

Table I

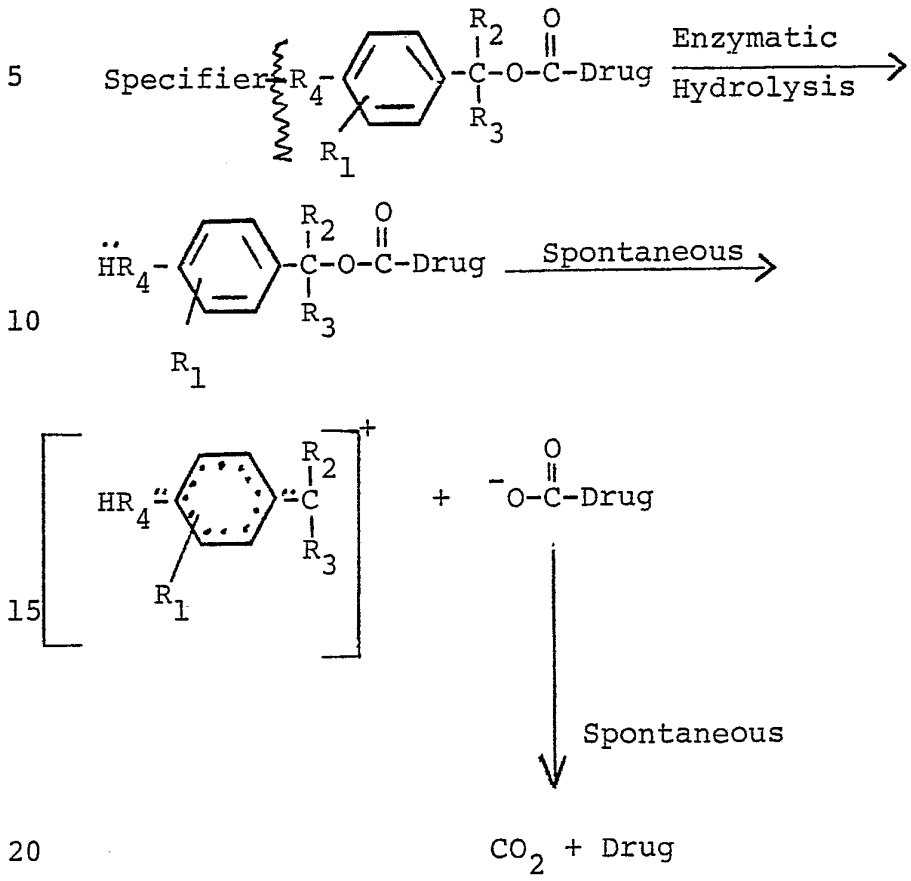
Specifier Moiety	R_4	Target Hydrolytic Enzyme
Peptide or amino acid	NH	Peptide hydrolase (protease)
Carboxylic acid	NH	Amidase
Phosphoric acid	NH	Phosphoramidase
Carboxylic acid	O	Carboxylic ester hydrolase
Phosphoric acid	O	Phosphoric monoesterase or diesterase
Sulfuric acid	O	Sulfuric ester hydrolase

The carbonate end of the connector moiety enables derivatization of either a free amino group (by forming a carbamate linkage), a free hydroxyl group (by forming a mixed carbonate linkage), or a free sulfhydryl group (by forming a mono-thio mixed carbonate linkage) on a drug molecule. A wide variety of different types of pharmacologically active agents having one or more of such reactive groups on their molecule will have their pharmacological activity inhibited by derivatization of such reactive groups, and hence would be suitable for use as the drug moiety of Formula I. Representative pharmacologically active agents falling in this category, in addition to the various antineoplastic agents previously listed, are set forth, together with their respective types of pharmacological activity and derivatizable sites for inhibition thereof, in Table 2, below.

Table 2

Drug	Pharmacological Activity	Derivatizable Site
Fluocinonide	Anti-psoriasas	C^{11}-OH
Betamethasone	Anti-rheumatiod arthritis	OH
Amantadine	Antiviral	NH_2
Aminocaproic acid	Hemostatic	NH_2
Oxytocin	Labor-inducing	NH_2
Isoniazid	Antibacterial, antitubercular	NH_2
Cycloserine	Antibacterial	NH_2
Methyl dopa	Antihypertensive	NH_2
Methimazole	Antithyroid hormone	SH
Anileridine	Analgetic	NH_2
Mestranol	Estrogen	C^{17}-OH
Phenelzine	Antidepressant	NH_2
Phenylpropanolamine	Adrenergic stimulant	NH_2

The theory underlying the mechanism of the self-immolative connector in accordance with the present invention, is explained by the following reaction scheme:

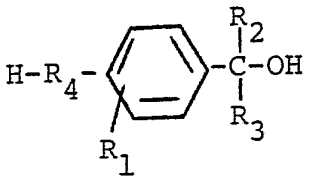


The specifier moiety and R_4 together act as a group with poor electron-donating capacity. However, enzymatic cleavage of the bond between the specifier moiety and R_4 by the target hydrolytic enzyme converts R_4 into a strongly electron-donating group, i.e., either NH_2 if R_4 is NH, or OH if R_4 is O. This electron donating effect greatly labilizes the benzylic bond to oxygen, which spontaneously ionizes. Spontaneous decarboxylation of the carbonate anion will then release the drug moiety in pharmacologically active form.

The overall release of the drug moiety from the tripartate pro-drug is determined by two processes, namely, (1) the rate of enzymatic hydrolysis of the bond linking the specifier moiety to R_4, and (2) the rate of ionization of the bond to the benzylic center. If R_1 in Formula I is an electron-donating group, this would tend to decrease process (1) and increase process (2). On the other hand, if R_1 is an electron-withdrawing group, this would tend to increase process (1) and decrease process (2). The net effect of R_1 on the rate of release of the drug moiety from the tripartate pro-drug may thus be relatively independent of whether R_1 releases or withdraws electrons. The system is thus at least partially buffered against the electronic nature of R_1, at least over a certain range. This means that R_1 can be chosen to be either relatively polar, for example, $-NH_2$ or $-NH^+(CH_3)_3$, or relatively non-polar, for example, $-C_6H_5$ or $-CH_3$, in order to alter the pharmocokinetic properties of the molecule as desired. The resulting tripartate molecules should not vary greatly in the rate of drug release once they equilibrate with the compartment where the target hydrolytic enzyme acts. R_1 may thus be chosen to optimize, for example, log P(1-octanol-water partition coefficient).

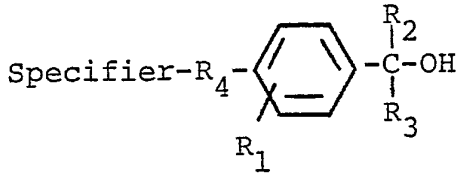
In regard to R_2 and R_3 in Formula I, the predominant effect of these groups will be on the rate of ionization of the bond to the benzylic center. Electron-donating substituents on these groups will tend to increase the ionization rate, while electron-withdrawing substituents on these groups will tend to decrease the ionization rate.

The hydrolytic enzyme-activatible tripartate pro-drugs in accordance with the present invention, may be readily synthesized by, first of all, reacting the specifier with a p-substituted benzyl alcohol reactant having the general formula



(II)

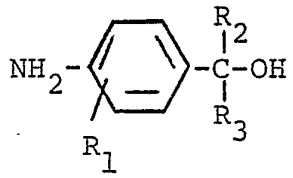
wherein R_1, R_2, R_3 and R_4 have the same meanings as defined above, to obtain a specifier-benzyl alcohol intermediate derivative having the general formula



(III)

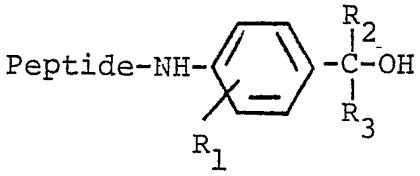
The specifier-benzyl alcohol intermediate derivative is then reacted with either phosgene or a chloroformate reagent, such as pentafluorophenyl chloroformate, pentachlorophenyl chloroformate, or p-nitrophenyl chloroformate, to form a second intermediate derivative. This second intermediate derivative will be either a specifier-benzyl chloroformate intermediate derivative, if the reactant is phosgene, or a specifier-benzyl mixed carbonate intermediate derivative, if the reactant is a chloroformate reagent. In either case, such second intermediate derivative is then reacted with the reactive site of the drug whose derivatization inhibits pharmacological activity (i.e., either a free amino group, a free hydroxyl group, or a free sulfhydryl group), to obtain the pro-drug of Formula I.

Applying the above-described general procedure to the synthesis of tripartate peptidyl derivative pro-drugs of antineoplastic agents in accordance with the present invention, the peptide specifier, as described in detail hereinabove,with its C-terminus in the free acid form and with all of its other reactive groups suitably blocked with protecting groups, is reacted at its C-terminus with the free amino group of a p-amino benzyl alcohol reactant having the general formula



(IV)

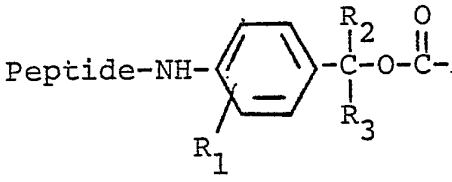
wherein R_1, R_2, R_3 and R_4 have the same meanings as defined above, to obtain a peptidyl benzyl alcohol having the general formula



(V)

This reaction is preferably carried out using a suitable condensing reagent, such as for example, N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline(EEDQ) in dimethylformamide, to avoid the necessity for protecting the benzylic alcohol function. The peptidyl benzyl alcohol is then reacted with either phosgene or a chloroformate reagent, such as pentafluorophenyl chloroformate, pentachlorophenyl chloroformate, or p-nitrophenyl chloroformate,to convert the peptidyl benzyl alcohol into either a peptidyl benzyl chloroformate (if phosgene is used as the reactant) or a peptidyl benzyl mixed carbonate (if a chloroformate reagent is used as the reactant). The peptidyl benzyl chloroformate or peptidyl benzyl mixed carbonate is

then reacted with a reactive site (a free amino group, a free hydroxyl group, or a free sulfhydryl group) of the antineoplastic agent whose derivatization inhibits pharmacological activity, to obtain a derivatization reaction product from which the protecting groups are then removed, such derivatization reaction product having the general formula



(VI)

In preparing the peptidyl derivative pro-drugs of antineoplastic agents in accordance with the present invention, the tripartate molecular structure of Formula VI, consisting of the peptide specifier moiety, intermediate self-immolative connective moiety, and the antineoplastic agent moiety, is particularly advantageous when the antineoplastic agent moiety is adriamycin; daunomycin, or bis-(2-chloroethyl) amine, since the molecules of these drugs are such as to tend to cause steric or electronic hindrance problems if the intended drug-activating enzymatic cleavage site is in close proximity to the drug molecule. These problems are minimized by the spacing provided by the self-immolative connector moiety.

The hydrolytic enzyme-activatible pro-drugs of the present invention, whether of the bipartate or tripartate molecular structure, will generally be administered in the same manner as the parent drug, i.e., orally or parenterally, with parenteral administration, e.g., intravenous, intramuscular or intraarterial, being generally preferred in order to minimize the possibility of premature activation of the pro-drug by non-specific hydrolytic enzymes. The dose levels of the pro-drug should be such

as to provide the requisite dose of the free drug. This will generally require that the pro-drug be administered in somewhat larger doses than the parent drug sufficient to allow for the possibility of incomplete activation of the pro-drug into the free drug.

The invention will be further illustrated by way of the following examples.

Example 1

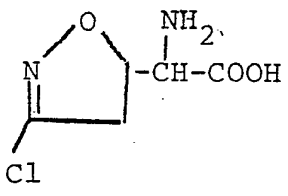
(A) Synthesis of Peptide Specifier

Boc-D-Val was condensed with Leu-OMe ester using dicyclohexylcarbodiimide (DCC) in dimethylformamide/methylene chloride solution. The resulting dipeptide was converted to the free acid by hydrolyzing the ester with NaOH to yield Boc-D-Val-Leu-OH.

N^{α}-CBZ-N^{ε}-Boc-Lys was converted to the methyl ester via treatment with CH_2N_2 and the amino group freed by hydrogenolysis over Pd/C to yield N^{ε}-Boc-Lys-OMe. This latter compound was condensed with the Boc-D-Val-Leu-OH (prepared as above) via a mixed anhydride reaction with isobutylchloroformate in dimethylformamide to yield Boc-D-Val-Leu-N^{ε}-Boc-Lys-OMe. The crude tripeptide was purified by column chromatography on silica gel, and the methyl ester freed by alkaline hydrolysis to yield Boc-D-Val-Leu-N^{ε}-Boc-Lys-OH.

(B) Synthesis of Bipartate Pro-Drug

The antineoplastic agent AT-125 having the formula



was derivatized at its free amino group by condensing it with the protected peptide specifier Boc-D-Val-Leu-N^{ε}-Boc-Lys-OH (prepared as above) via a mixed anhydride reaction with isobutyl-chloroformate in dioxane/tetrahydrofuran. The resulting product was deprotected with trifluoro-acetic acid (TFA) in methylene chloride to yield the desired pro-drug D-Val-Leu-Lys-AT-125.

A portion of this peptidyl derivative pro-drug was treated with trypsin in Tris buffer. Two products were obtained which showed R_f on TLC silica plates corresponding to the tri-peptide D-Val-Leu-Lys-OH and the free drug AT-125. This demonstrates that the pro-drug is a sub-strate for trypsin and, presumably, other trypsin-like proteases such as plasmin and plasminogen activator, which generally show a specificity similar to that of trypsin. A similar result was obtained with plasmin.

Example 2

The peptidyl derivative pro-drug prepared in accordance with Example 1, was tested for its tumor-specific cytotoxic activity by means of an in vitro test procedure utilizing a cell culture system with well-matched normal and malignant cells which differed substantially in fibrinolytic enzyme (i.e., plasmin and plasminogen activator) levels but displayed similar good sensitivity to the free drug. The cell culture system employed was chick embryo fibroblasts, both normal and transformed with Rous Sarcoma virus. The transformed cells exhibit a substantially higher level of fibrinolytic enzyme activity than the normal cells. The test procedure was carried out as follows.

Chick embryo fibroblasts, either normal (N) or transformed with Rous Sarcoma virus (SR) were plated in 35 mm plastic dishes at an initial titer of 1.5 x 10^5 cells per dish (N) or 3 x 10^5 cells per dish (SR). Allowing for the difference in plating efficiencies between normal and transformed cells, this leads to similar numbers of cells per plate. The medium used was Dulbecco's Modified Minimal Eagle's Medium (DME) supplemented with 10% Tryptose-Phosphate broth, 4% calf serum, and 1% chick serum. Cells grow exponentially in this medium with a doubling time of 18 to 20 hours. After 24 hours the cells were changed to DME medium containing 5% chick serum and lacking Tryptose-Phosphate broth and calf serum. After a further 18 hours, the test drugs (which included both the peptidyl derivative pro-drug and, for purposes of comparison, the underivatized parent drug) were added at various concentrations for a further 5 hours. Finally, in order to measure DNA synthesis ^{3}H-thymidine at a final concentration of 1 microcurie/ml was added to each dish for 30 minutes. The medium was removed and the cells were incubated with cold 5% trichloroacetic acid for a further 15 minutes. After 3 further washes with cold trichloroacetic acid, the DNA was hydrolyzed with 10% trichloroacetic acid at 70°C for two hours. The solubilized material was counted in an Omnifluor-Triton-X-100 scintillation fluid. Control experiments have demonstrated that this measurement of thymidine incorporation into DNA corresponds to cell numbers as determined in a Coulter counter.

By means of plotting the residual ^{3}H-thymidine incorporation as a function of drug concentration compared to untreated control, the ED_{50} (i.e., the drug concentration at which incorporation of thymidine

is reduced to 50% of the untreated control) is determined for each of the two test drugs (i.e., the peptidyl derivative pro-drug and the corresponding underivatized parent drug) against both the normal cells and the transformed cells. The therapeutic index of each drug (i.e., the ratio of its pharmacological activity to its toxicity) is then determined as the ratio of its ED_{50} against the normal cells to its ED_{50} against the transformed cells. Following this procedure, the therapeutic index for the peptidyl derivative pro-drug in accordance with the present invention was determined to be 5.3, in comparison to a therapeutic index of 1.2 for the corresponding underivatized parent drug. Thus, the peptidyl derivative pro-drug in accordance with the present invention exhibits an approximately 5-fold improvement in therapeutic index over the corresponding underivatized parent drug.

The above-described test results are indicative of the fact that the peptidyl derivative pro-drugs of the present invention are selective substrates for drug-activating enzymatic cleavage by tumor-associated fibrinolytic enzymes,and are selectively activatible to release cytotoxic levels of pharmacologically active drug at sites exhibiting elevated levels of such fibrinolytic enzyme activity.

Example 3

Employing a synthesis procedure similar to that described in Example 1, above, the antineoplastic agent, phenylenediamine mustard, was derivatized at its free amino group with D-Val-Leu-Lys peptide specifier to convert it into the peptidyl derivative pro-drug D-Val-Leu-Lys-phenylenediamine mustard.

When tested by means of the in vitro assay described in Example 2, above, the D-Val-Leu-Lys-phenylenediamine mustard pro-drug showed a 7-fold improvement in its therapeutic index in comparison with the underivatized phenylenediamine mustard parent drug.

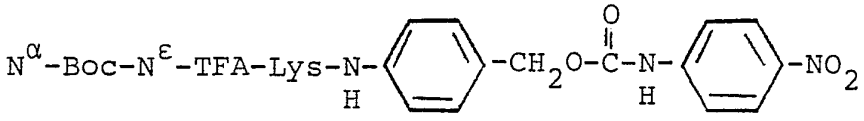
Example 4

Employing a synthesis procedure similar to that described in Example 1, above, the antineoplastic agent, adriamycin, was derivatized at its free amino group on daunosamine with the D-Val-Leu-Lys peptide specifier to convert it into D-Val-Leu-Lys-adriamycin pro-drug. When tested by means of the in vitro assay described in Example 2, above,the D-Val-Leu-Lys-adriamycin pro-drug showed a 6-fold improvement in its therapeutic index, in comparison with the underivatized adriamycin parent drug.

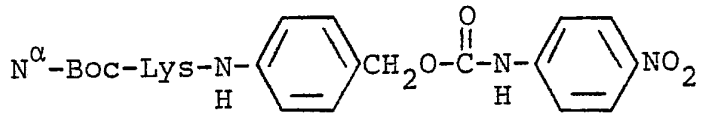
Example 5

The feasibility of the self-immolative connector aspect of the present invention in the design of hydrolytic enzyme-activatible tripartate pro-drugs was demonstrated by means of the following model study which utilized p-nitroaniline as the "drug" because of the ease of colorimetric detection.

Synthesis of the tripartate pro-drug model was carried out as follows. N^{α}-t-Boc-N^{ε}-TFA lysine was coupled to p-aminobenzyl alcohol using N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) in dimethyl-formamide. The product was purified by crystallization from ethyl acetate:ether. Next this material was reacted with p-nitrophenylisocyanate in dry pyridine to yield



which was purified by preparative TLC on silica. Finally, the TFA group was removed with tetramethylguanidine in acetonitrile:water 1:1 to yield the tripartate pro-drug model having the formula



The final structure was characterized by NMR and IR spectroscopy.

In order to demonstrate the possibility of self-immolation with this tripartate pro-drug model, it was dissolved in 1 ml 50 mM Bis-Tris-Cl buffer pH 6.7 at 1 mM and treated with 2 µg of trypsin. There was an instantaneous release of p-nitroaniline as measured by the increase of optical density at 405 nm. The rate of release of p-nitroaniline was comparable to the rate of release expected for cleavage of the Lys-anilide bond, and no lag was noted. TLC analysis showed the expected products N^{α}-Boc-Lys-OH, p-aminobenzyl alcohol, and p-nitroaniline. The release of p-nitroaniline was blocked by prior treatment of trypsin with tosyl-lysyl chloromethyl ketone (TLCK).

Two similar tripartate pro-drug models were similarly synthesized, one with one of the benzylic hydrogens replaced by a methyl group, and the other with p-nitroaniline replaced by aniline. In both cases, TLC analysis again showed that trypsin hydrolysis released the expected products,including p-nitroaniline or aniline. This was confirmed spectrophotometrically in the case of the p-nitroaniline derivative.

The above-described model studies demonstrate the feasibility under physiological conditions of the self-immolation of the intermediate connector moiety in the hydrolytic enzyme-activatible tripartate pro-drugs in accordance with the present invention.

While the fibrinolytic and blood-coagulating enzyme-activatible peptidyl derivative pro-drugs in accordance with the present invention have been described with particular reference to the preferred embodiments thereof wherein the drug moiety is an antineoplastic agent, it will be understood that the drug moiety, in either the bipartate or tripartate structure, could be any normally pharmacologically active compound which is suitably convertible into a pro-drug by derivatization with the peptide specifiers described above and whose site of intended action is known to exhibit elevated levels of fibrinolytic and/or blood-coagulating enzyme activity. By way of example, elevated levels of fibrinolytic and/or blood-coagulating enzymes are normally exhibited in the skin, which is the site of intended action of anti-psoriasis agents, such as fluocinonide; in the joint, which is the site of intended action of anti-arthritic agents, such as betamethasone; and in the uterus, which is the site of intended action of antifertility or implantation agents such as estrogenic and progestational steroids. Any of these drugs could be suitably derivatized with the peptide specifiers as described above to convert them into peptidyl pro-drugs, of either the bipartate or tripartate structure, which are selective substrates for fibrinolytic and/or blood-coagulating proteases so as to be selectively activatible at their site of intended action.

CLAIMS

1. A tumor-specific pro-drug of an antineoplastic agent comprising a peptidyl derivative of said antineoplastic agent, said peptidyl derivative being a selective substrate for drug-activating enzymatic cleavage by one or more tumor-associated proteases selected from the group consisting of fibrinolytic enzymes and blood-coagulating enzymes.

2. The pro-drug of Claim 1, wherein said tumor-associated protease is a fibrinolytic enzyme selected from the group consisting of plasmin and plasminogen activator.

3. The pro-drug of Claim 1, wherein said tumor-associated protease is a blood-coagulating enzyme selected from the group consisting of thrombin, thromboplastin, Factor Va, Factor VIIa, Factor VIIIa, Factor IXa, Factor Xa, Factor XIa, and Factor XIIa.

4. The pro-drug of Claim 1, wherein the molecular structure of said peptidyl derivative is comprised of a peptide specifier moiety and an antineoplastic agent moiety, said two moieties being covalently linked together either directly or through an intermediate self-immolative connector moiety in a manner such that said antineoplastic agent moiety is rendered pharmacologically inactive, the site of said drug-activating enzymatic cleavage will be at the bond covalently linking said peptide specifier moiety to its immediately adjacent moiety, and said drug-activating enzymatic cleavage will effect release of said antineoplastic agent moiety in pharmacologically active form.

5. The pro-drug of Claim 4, wherein the covalent linkage of said peptide specifier moiety to its immediately adjacent moiety is at the C-terminus of said peptide specifier moiety, and the C-terminal amino acid residue of said peptide specifier moiety is a basic amino acid residue.

6. The pro-drug of Claim 5, wherein said C-terminal amino acid residue is arginine or lysine.

7. The pro-drug of Claim 5, wherein said peptide specifier moiety contains a hydrophobic amino acid residue or glycine in the position immediately adjacent to said C-terminal amino acid residue.

8. The pro-drug of Claim 7, wherein said hydrophobic amino acid residue is selected from the group consisting of alanine, valine, leucine, isoleucine, methionine, phenylalanine, tryptophan and proline.

9. The pro-drug of Claim 5, wherein the N-terminal amino acid residue of said peptide specifier moiety is a D-amino acid residue, a protected L-amino acid residue, or protected glycine.

10. The pro-drug of Claim 5, wherein said peptide specifier moiety has an amino acid residue chain length ranging from that of a tripeptide to that of a pentadecapeptide.

11. The pro-drug of Claim 10, wherein said peptide specifier moiety contains a hydrophobic amino acid residue or glycine in the position immediately adjacent to said C-terminal amino acid residue, and the N-terminal amino acid residue of said peptide specifier moiety is a D-amino acid residue, a protected L-amino acid residue or protected glycine.

12. The pro-drug of Claim 10, wherein the amino acid residue sequence in said peptide specifier moiety is such that its C-terminal amino acid residue is arginine or lysine, its amino acid residue in the position immediately adjacent to said C-terminal amino acid residue is alanine, leucine, or glycine, and its N-terminal amino acid residue is a D-amino acid residue, a protected L-amino acid residue or protected glycine.

13. The pro-drug of Claim 12, wherein said C-terminal amino acid residue is lysine, said amino acid residue in the position immediately adjacent to said C-terminal amino acid residue is leucine, and said N-terminal amino acid residue is D-valine or D-isoleucine.

14. The pro-drug of Claim 12, wherein the amino acid residue sequence in said peptide specifier moiety substantially mimics the amino acid residue sequence on the carboxyl side of the Arg-Val bond in plasminogen which serves as the site of cleavage of plasminogen by plasminogen activator, with said C-terminal amino acid residue being arginine, and said amino acid residue in the position immediately adjacent to said C-terminal amino acid residue being glycine.

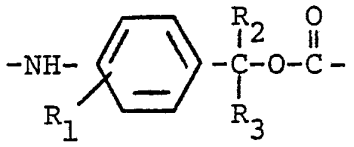
15. The pro-drug of Claim 4, wherein said peptidyl derivative has a bipartate molecular structure consisting of said peptide specifier moiety and said antineoplastic agent moiety, said two moieties being directly covalently linked together by means of a covalent linkage formed between the C-terminus of said peptide specifier moiety and a carboxyl-reactive site of said antineoplastic agent moiety whose derivatization inhibits pharmacological activity.

16. The pro-drug of Claim 15, wherein said covalent linkage is an amide linkage formed between the C-terminus of said peptide specifier moiety and a free amino group of said antineoplastic agent moiety.

17. The pro-drug of Claim 15, wherein said covalent linkage is an ester linkage formed between the C-terminus of said peptide specifier moiety and a free hydroxyl group of said antineoplastic agent moiety.

18. The pro-drug of Claim 4, wherein said peptidyl derivative has a tripartate molecular structure consisting of said peptide specifier moiety, said intermediate self-immolative connector moiety and said antineoplastic agent moiety, said intermediate self-immolative connector moiety being covalently linked at its one end to the C-terminus of said peptide specifier moiety and covalently linked at its other end to a reactive site of said antineoplastic agent moiety whose derivatization inhibits pharmacological activity, said intermediate self-immolative connector moiety having a molecular structure such that said drug-activating enzymatic cleavage of the bond covalently linking it to said peptide specifier moiety will initiate spontaneous cleavage of the bond covalently linking it to said antineoplastic agent moiety to thereby effect release of said antineoplastic agent moiety in pharmacologically active form.

19. The pro-drug of Claim 18, wherein said intermediate self-immolative connector moiety has the general formula:



wherein R_1 is hydrogen or one or more substituent groups which are either electron-donating groups or electron-withdrawing groups, and R_2 and R_3 may be the same or different and are each selected from the group consisting of hydrogen, alkyl , phenyl, and phenyl substituted with either electron-donating groups or electron-withdrawing groups, said connector moiety having its terminal amino group covalently linked to the C-terminus of said peptide specifier moiety and its terminal carbonyl group covalently linked to said reactive site of said antineoplastic agent moiety.

20. The pro-drug of Claim 19, wherein R_1 is hydrogen, R_2 is hydrogen or methyl, and R_3 is hydrogen or methyl.

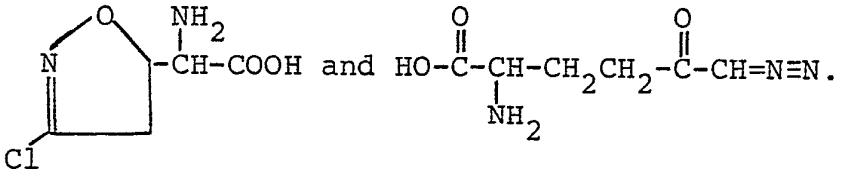
21. The pro-drug of Claim 19, wherein said reactive site of said antineoplastic agent moiety is a free amino group, a free hydroxyl group, or a free sulfhydryl group.

22. The pro-drug of Claim 21, wherein said antineoplastic agent moiety is selected from the group consisting of adriamycin, daunomycin, and bis-(2-chloroethyl) amine.

23. The pro-drug of Claim 4, wherein the covalent linkage of said antineoplastic agent moiety to its immediately adjacent moiety is at a reactive site of said antineoplastic agent moiety whose derivatization inhibits pharmacological activity.

24. The pro-drug of Claim 23, wherein said reactive site of said antineoplastic agent moiety is a free amino group, a free hydroxyl group, or a free sulfhydryl group.

25. The pro-drug of Claim 24, wherein said antineoplastic agent moiety is selected from the group consisting of cytosine arabinoside, adriamycin, daunomycin, 6-thioguanine, fluorodeoxyuridine, bis-(2-chloroethyl) amine, phenylenediamine mustard, 3'-amino-thymidine, L-alanosine, 2-aminothiodiazole, 1,4-dihydroxy-5,8-bis(2-aminoethylamino)-9,10-anthracenedione,



and

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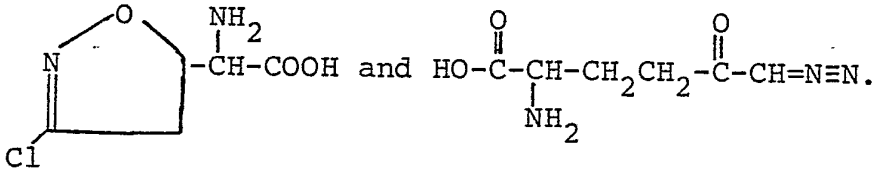
26. The pro-drug of Claim 25, wherein said peptide specifier moiety has an amino acid residue chain length ranging from that of a tripeptide to that of a pentadecapeptide, the covalent linkage of said peptide specifier moiety to its immediately adjacent moiety is at the C-terminus of said peptide specifier moiety, and the amino acid residue sequence in said peptide specifier moiety is such that its C-terminal amino acid residue is a basic amino acid residue, its amino acid residue in the position immediately adjacent to said C-terminal amino acid residue is a hydrophobic amino acid residue or glycine, and its N-terminal amino acid residue is a D-amino acid residue, a protected L-amino acid residue or protected glycine.

27. A method of rendering an antineoplastic agent tumor-specific which comprises derivatizing said antineoplastic agent either directly or through an intermediate self-immolative connector with a peptide specifier at a reactive site appropriate for inhibiting the pharmacological activity of said antineoplastic agent, said peptide specifier having an amino acid residue sequence such that it will be selectively enzymatically cleaved from said antineoplastic agent by one or more tumor-associated proteases selected from the group consisting of fibrinolytic enzymes and blood-coagulating enzymes so as to effect release of said antineoplastic agent in pharmacologically active form.

28. The method of Cliam 27, wherein said tumor-associated protease is a fibrinolytic enzyme selected from the group consisting of plasmin and plasminogen activator.

29. The method of Claim 27, wherein said reactive site is a free amino group, a free hydroxyl group, or a free sulfhydryl group.

30. The method of Claim 29, wherein said antineoplastic agent is selected from the group consisting of cytosine arabinoside, adriamycin, daunomycin, 6-thioguanine, fluorodeoxyuridine, bis-(2-chloroethyl) amine, phenylenediamine mustard, 3'-aminothymidine, L-alanosine, 2-aminothiodiazole, 1,4-dihydroxy-5,8-bis (2-aminoethylamino)-9,10-anthracenedione,



and

.

31. The method of Claim 27, wherein said derivatization is carried out either directly or through an intermediate self-immolative connector with the C-terminus of said peptide specifier, and the C-terminal amino acid residue of said peptide specifier is a basic amino acid residue.

32. The method of Claim 31, wherein said peptide specifier has an amino acid residue chain length ranging from that of a tripeptide to that of a pentadecapeptide, and its N-terminal amino acid residue is a D-amino acid residue, a protected L-amino acid residue or protected glycine.

33. The method of Claim 32, wherein said peptide specifier contains a hydrophobic amino acid residue or glycine in the position immediately adjacent to said C-terminal amino acid residue.

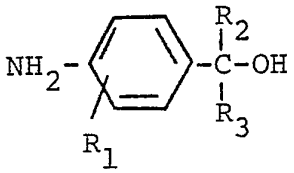
34. The method of Claim 33, wherein said C-terminal amino acid residue is lysine, said amino acid residue in the position immediately adjacent to said C-terminal amino acid residue is leucine, and said N-terminal amino acid residue is D-valine or D-isoleucine.

35. The method of Claim 33, wherein the amino acid residue sequence in said peptide specifier substantially mimics the amino acid residue sequence on the carboxyl side of the Arg-Val bond in plasminogen which serves as the site of cleavage of plasminogen by plasminogen activator, with said C-terminal amino acid residue being arginine, and the amino acid residue in the position immediately adjacent to said C-terminal amino acid residue being glycine.

36. The method of Claim 27, wherein the peptide specifier reactant in the derivatization reaction has its C-terminus in the free acid form and all of its other reactive groups suitably blocked with protecting groups, said reactive site is a free amino group or a free hydroxyl group of said antineoplastic agent, said derivatization reaction is carried out directly between said C-terminus of said peptide specifier reactant and said reactive site of said antineoplastic agent, and subsequent to said derivatization reaction said protecting groups are removed from the reaction product.

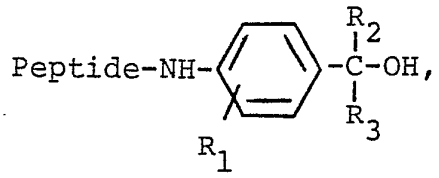
37. The method of Claim 27, wherein the peptide specifier reactant in the derivatization reaction has its C-terminus in the free acid form and all of its other reactive groups suitably blocked with protecting groups; said reactive site is a free amino group, a free hydroxyl group or a free sulfhydryl group of said antineoplastic agent; said derivatization reaction is carried out in a multi-step procedure comprising:

(a) reacting said C-terminus of said peptide specifier reactant with the free amino group of a p-aminobenzyl alcohol reactant having the general formula



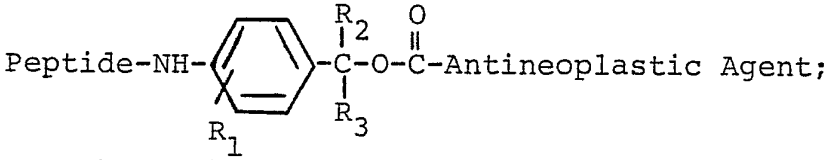
wherein R_1 is hydrogen or one or more substituent groups which are either electron-donating groups or electron-withdrawing groups, and R_2 and R_3 may be the same or different

and are each selected from the group consisting of hydrogen, alkyl, phenyl, and phenyl substituted with either electron-donating groups or electron-withdrawing groups, to obtain a peptidyl benzyl alcohol having the general formula



(b) reacting said peptidyl benzyl alcohol with either phosgene or a chloroformate reagent to convert said peptidyl benzyl alcohol into, respectively, either a peptidyl benzyl chloroformate or a peptidyl benzyl mixed carbonate, and

(c) reacting said peptidyl benzyl chloroformate or peptidyl benzyl mixed carbonate with said reactive site of said antineoplastic agent to obtain a derivatization reaction product having the general formula



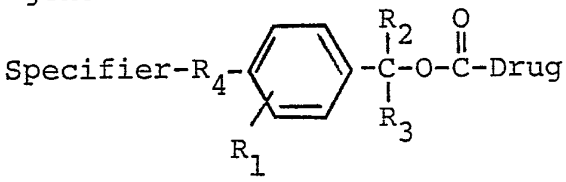
and subsequent to said derivatization reaction said protecting groups are removed from said derivatization reaction product.

38. The method of Claim 37, wherein R_1 is hydrogen, R_2 is hydrogen or methyl, and R_3 is hydrogen or methyl.

39. The method of Claim 37, wherein said chloroformate reagent is selected from the group consisting of pentafluorophenyl chloroformate, pentachlorophenyl chloroformate, and p-nitrophenyl chloroformate.

40. In a method for the treatment of neoplastic diseases in an animal or human host which comprises administering to said host in need thereof an antineoplastically effective amount of an antineoplastic agent, the improvement consisting of administering said antineoplastic agent in the form of the peptidyl derivative pro-drug of Claim 1, whereby the selectivity of delivery of said antineoplastic agent to the tumor site of intended action is enhanced.

41. A hydrolytic enzyme-activatible pro-drug having the general formula



wherein R_1 is hydrogen or one or more substituent groups which are either electron-donating groups or electron-withdrawing groups; R_2 and R_3 may be the same or different and are each selected from the group consisting of hydrogen, alkyl, phenyl, and phenyl substituted with either electron-donating groups or electron-withdrawing groups; R_4 is NH or O; when R_4 is NH, the specifier moiety is selected from the group consisting of a peptide, an amino acid, a carboxylic acid, and phosphoric acid; when R_4 is O, the specifier moiety is selected from the group consisting of a carboxylic acid, phosphoric acid, and sulfuric acid; and the drug moiety is a normally pharmacologically active agent having a reactive site whose derivatization inhibits pharmacological activity, said reactive site being selected from the group consisting

of a free amino group, a free hydroxyl group and a free sulfhydryl group, the covalent linkage between said drug moiety and its adjacent carbonyl group being at said reactive site so as to inhibit the pharmacological activity of said drug moiety.

42. The pro-drug of Claim 41, wherein R_1 is hydrogen, R_2 is hydrogen or methyl, and R_3 is hydrogen or methyl.

43. The pro-drug of Claim 41, wherein said drug moiety is an antineoplastic agent.

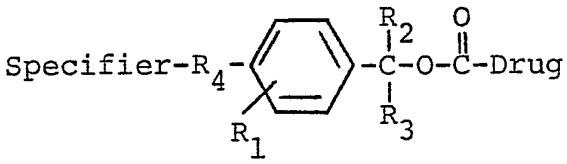
44. The pro-drug of Claim 41, wherein R_4 is NH and the specifier moiety is a peptide.

45. The pro-drug of Claim 42, wherein said drug moiety is an antineoplastic agent, R_4 is NH and the specifier moiety is a peptide.

46. In a method of converting a pharmacologically active drug into a hydrolytic enzyme-activatible pro-drug by derivatizing said drug at a reactive site thereof appropriate for inhibiting its pharmacological activity, with a specifier designed to be selectively enzymatically cleaved from the resulting pro-drug by a target hydrolytic enzyme so as to effect release of said drug in pharmacologically active form, the improvement consisting of spacing the specifier moiety from the drug moiety by means of an intermediate self-immolative connector moiety covalently linked at its one end to said specifier moiety and covalently linked at its other end to said reactive site of said drug moiety, said intermediate self-immolative connector moiety having a molecular structure such that the drug-activating enzymatic cleavage of the bond covalently linking it to said specifier moiety will initiate spontaneous cleavage of the bond covalently linking it to said drug moiety to thereby effect release of said drug in pharmacologically active form.

47. The method of Claim 46, wherein said reactive site of said drug is selected from the group consisting of a free amino group, a free hydroxyl group, and a free sulfhydryl group,and said specifier is selected from the group consisting of a peptide, an amino acid, a carboxylic acid, phosphoric acid and sulfuric acid.

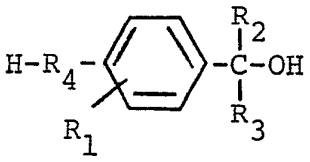
48. The method of Claim 47, wherein said resulting pro-drug has the general formula



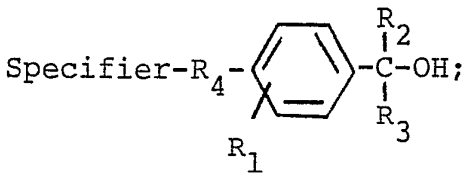
wherein R_1 is hydrogen or one or more substituent groups which are either electron-donating groups or electron-withdrawing groups; R_2 and R_3 may be the same or different and are each selected from the group consisting of hydrogen, alkyl, phenyl, and phenyl substituted with either electron-donating groups or electron-withdrawing groups; R_4 is NH or O; when R_4 is NH, the specifier moiety is selected from the group consisting of a peptide, an amino acid, a carboxylic acid, and phosphoric acid; and when R_4 is O, the specifier moiety is selected from the group consisting of a carboxylic acid, phosphoric acid, and sulfuric acid.

49. The method of Claim 48, wherein the preparation of said pro-drug comprises the steps of:

(a) reacting said specifier with a p-substituted benzyl alcohol reactant having the general formula



wherein R_1, R_2, R_3 and R_4 have the same meanings as defined above, to obtain a specifier-benzyl alcohol intermediate derivative having the general formula



(b) reacting said specifier-benzyl alcohol intermediate derivative with either phosgene or a chloroformate reagent to convert said specifier-benzyl alcohol intermediate derivative into, respectively, either a specifier-benzyl chloroformate intermediate derivative or a specifier-benzyl mixed carbonate intermediate derivative; and

(c) reacting said specifier-benzyl chloroformate intermediate derivative or specifier-benzyl mixed carbonate intermediate derivative with said reactive site of said drug to obtain said pro-drug.

50. The method of Claim 49, wherein said chloroformate reagent is selected from the group consisting of pentafluorophenyl chloroformate, pentachlorophenyl chloroformate, and p-nitrophenyl chloroformate.

51. The method of Claim 49, wherein R_1 is hydrogen, R_2 is hydrogen or methyl, and R_3 is hydrogen or methyl.

52. The method of Claim 49, wherein said drug is an antineoplastic agent.

53. The method of Claim 49, wherein R_4 is NH and the specifier is a peptide.

54. The method of Claim 51, wherein said drug is an antineoplastic agent, R_4 is NH, and the specifier is a peptide.

55. A pro-drug of a normally pharmacologically active compound comprising a peptidyl derivative of said compound, said peptidyl derivative being a selective substrate for drug-activating enzymatic cleavage for one or more proteases selected from the group consisting of fibrinolytic enzymes and blood-coagulating enzymes.

56. The pro-drug of Claim 55, wherein said protease is a fibrinolytic enzyme selected from the group consisting of plasmin and plasminogen activator.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US80/01290

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

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

IPC: [3] C07C 103/52 , A61K 37/00
C08L 37/00,

US. CLASS. 260-112.5R, 424/177
260-112.R

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
US	260-112.5R, 260-112.R, 424-177

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
X P	US, A, 4,192,799 Published 11 March 1980	1-56
X	US, A, 4,119,589 Published 10 October 1978	1-56
X	US, A, 4,123,519 Published 31 October 1978	1-56
X	US, A, 4,119,589 Published 10 October 1978	1-56
X	US, A, 4,140,679 Published 20 February 1979	1-56
X P	US, A, 4,193,982 Published 18 March 1980	1-56
X	US, A, 4,238,389 Published 9 December 1980	1-56
X	US, A, 4,039,519 Published 2 August 1977	1-56
X P	US, A, 4,223,013 Published 16 September 1980	1-56

* Special categories of cited documents: [15]

"A" document defining the general state of the art

"E" earlier document but published on or after the international filing date

"L" document cited for special reason other than those referred to in the other categories

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but on or after the priority date claimed

"T" later document published on or after the international filing date or priority date and not in conflict with the application, but cited to understand the principle or theory underlying the invention

"X" document of particular relevance

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
21 January 1981	28 JAN 1981
International Searching Authority [1]	Signature of Authorized Officer [20]
ISA/US	Robert K. Phillips

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

X	US,	A,	4,057,685 Published 8 November 1977	1-56
X	US,	A,	4,150,105 Published 17 April 1979	1-56
X	US,	A,	4,016,146 Published 5 April 1977	1-56
XP	US,	A,	4,201,770 Published 6 May 1980	1-56
X	US,	A,	4,009,264 Published 22 February 1977	1-56

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [10]

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

1. ☐ Claim numbers ....., because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers ....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [11]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

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

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

Remark on Protest

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

☐ No protest accompanied the payment of additional search fees.