

(12) PATENT ABRIDGMENT (11) Document No. AU-B-75564/87
(19) AUSTRALIAN PATENT OFFICE (10) Acceptance No. 614263

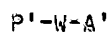
- (54) Title
IMMUNOTOXINS CONTAINING TRICHOSANTHIN OR TRICHOKIRIN GROUPS
- International Patent Classification(s)
(51)⁴ **C07K 015/12 A61K 039/44 C12P 021/08**
- (21) Application No. : **75564/87** (22) Application Date : **13.07.87**
- (30) Priority Data
- (31) Number (32) Date (33) Country
86 10297 15.07.86 FR FRANCE
- (43) Publication Date : **21.01.88**
- (44) Publication Date of Accepted Application : **29.08.91**
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- (56) Prior Art Documents
AU 43646/85
FR 74279

(57) The present invention relates to products, belonging to the class of the immunotoxins, which are obtained by the covalent coupling of, on the one hand, trichosanthin or trichokirin, as such or appropriately modified (denoted hereafter by the symbol A), with, on the other hand, an antibody or antibody fragment, used in its natural form or correctly modified (denoted hereafter by the symbol P), which is capable of selectively recognizing an antigen carried by the target cells to be destroyed. The 2 proteins can be coupled either via a disulfide bond or via a thioether bond.

Thus, according to one of its features, the present invention relates to an immunotoxin formed by the coupling of an antibody P with trichosanthin or trichokirin - proteins extracted from the plant *Trichosanthes kirilowii*

CLAIM

1. An immunotoxin formed by the coupling of an antibody with trichosanthin or trichokirin, having the following statistical formula ^{as hereinbefore defined}:



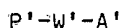
in which P' represents the radical of a protein P which is an

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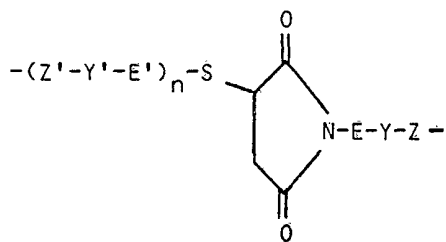
antibody or antibody fragment, as such or appropriately chemically modified, from which one or more of its ~~own~~ ^{functional} groups have been removed and in which other functional groups are optionally blocked, A' represents the radical of a protein A which is trichosanthin or trichokirin, as such or appropriately chemically modified, from which one or more of its ~~own~~ ^{functional} groups have been removed and in which other functional groups are optionally blocked, and W represents a divalent covalent structure containing at least one thioether group or one disulfide group in which either one of the sulfur atoms is either selected among the ones of the cysteines of P or A or is bonded to the groups belonging to P and/or A by spacing structures carrying a functional group bonded to the said groups belonging to P and A.

3. Immunotoxin formed by the coupling of an antibody with trichokirin or trichosanthin, having the following statistical formula :

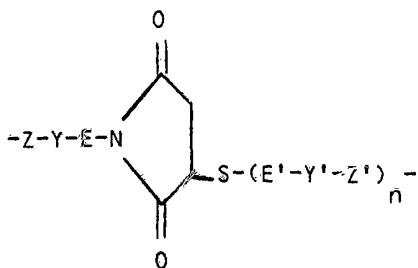


in which P' and A' are as defined in claim 1 and W' represents a covalent structure selected from the group consisting of :

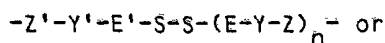
(a) a group of the formula :



(b) a group of the formula :



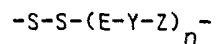
(c) a group of the formula :



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(d) a group of the formula :



in which :

- Z and Z', which are identical or different, represent the groups belonging to the proteins A and P, chosen from the oxygen atom originating from the hydroxyl of one of the tyrosine residues, the carbonyl group originating from one of the terminal carboxyls or the free carboxyls of the aspartic and/or glutamic acids of A and P, the group originating from the dialdehyde structure obtained after oxidation of the carbohydrate structure of P or optionally of the protein A with periodic acid, and the -NH- group originating from one of the terminal amines of A and P or from one of the amines in the epsilon position of one of the lysine residues ;
- Y and Y' represent groups covalently bonded to any one of the groups Z and Z' of the proteins A and P ;
- E and E' represent inert spacing structures ; and
- n represents zero or 1.

The present invention relates to products, belonging to the class of the immunotoxins, which are obtained by the covalent coupling of, on the one hand, trichosanthin or trichokirin, as such or appropriately modified (denoted hereafter by the symbol A), with, on the other hand, an antibody or antibody fragment, used in its natural form or correctly modified (denoted hereafter by the symbol P), which is capable of selectively recognizing an antigen carried by the target cells to be destroyed. The 2 proteins can be coupled either via a disulfide bond or via a thioether bond.

Thus, according to one of its features, the present invention relates to an immunotoxin formed by the coupling of an antibody P with trichosanthin or trichokirin - proteins extracted from the plant *Trichosanthos kirilowii* - having the following statistical formula:

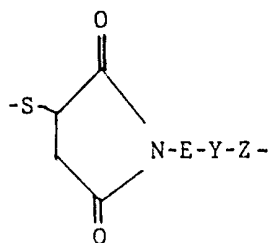


in which P' represents the radical of a protein which is an antibody or antibody fragment, as such or appropriately chemically modified, from which at least one of its ~~own~~^{functional} groups has been removed and in which the other functional groups are optionally blocked, A' represents the radical of a protein which is trichosanthin or trichokirin, as such or appropriately chemically modified, from which at least one of its ~~own~~^{functional} groups has been removed and in which the other functional groups are optionally blocked, and W represents a divalent covalent structure containing at least one thioether group or one disulfide group in which either one of the sulfur atoms is either selected among the ones of the cysteines of P or A or bonded to the groups belonging to P and/or A by spacing structures carrying a functional group bonded to the said groups



belonging to P and/or A.

A thioether bond between two proteins is understood as meaning a bond of the type:



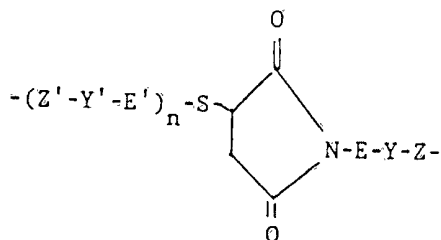
5 in which Z, Y and E are as defined below.

The present invention relates preferentially to an immunotoxin of the statistical formula:

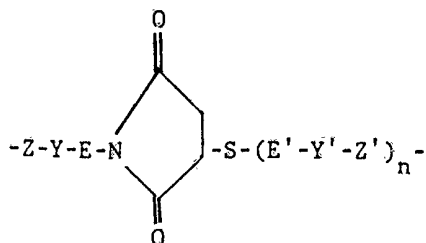


10 in which P' and A' are as defined above and W' represents a covalent structure chosen from:

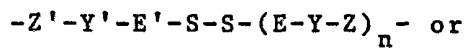
(a) a group of the formula:



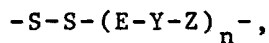
(b) a group of the formula:



(c) a group of the formula :



(d) a group of the formula :



5 in which

- Z and Z', which are identical or different, represent the groups belonging to the proteins A and P, chosen from the oxygen atom originating from the hydroxyl of one of the tyrosine residues, the carbonyl group
10 originating from one of the terminal carboxyls or the free carboxyls of the aspartic and/or glutamic acids of A and P, the group originating from the dialdehyde structure obtained after oxidation of the carbohydrate structure of P or optionally of the protein A with
15 periodic acid, and the -NH- group originating from one of the terminal amines of A and P or from one of the amines in the epsilon position of one of the lysine residues ;

- Y and Y' represent groups covalently bonded to any one
20 of the groups Z and Z' of the proteins A and P ;

- E and E' represent inert spacing structures ; and

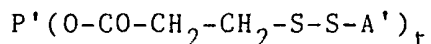
- n represents zero or 1.

As hereinafter defined the
The immunotoxins of the present invention are represented in simplified form by the *Statistical* formulae (I) and (II)
25 above, but it is understood that there can be several structures W or W' bonded to the same molecule of P and/or A, and hence several A's bonded to a single P and vice-versa, the number of bridges depending on the coupling process and the number of groups belonging to P
30 and A. Thus, the statistical formulae I and II also represent these products and mixtures thereof, which



correspond to the formula $P'(W'-A')_m$, in which m is an integer or fraction less than or greater than 1.

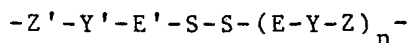
For example, if an immunotoxin is formed by the coupling of trichosanthin or trichokirin with the antibody P (for example the antibody T101) via a divalent covalent structure having a disulfide group in which one sulfur is that belonging to a cysteine of trichosanthin or trichokirin and the other is bonded to the phenolic oxygens of the tyrosines of the antibody P by an oxopropyl group, it will have the statistical formula:



in which t represents the number of tyrosines in the antibody (for example the antibody T101) which are involved in the coupling.

The resulting immunotoxin corresponds to a product of the formula (II) in which:

- P' is as defined above, especially the radical of the antibody T101 from which t phenolic groups of its tyrosines have been removed;
- A' is as defined above, especially the radical of trichosanthin or trichokirin from which the thiol group of one of its cysteines has been removed; and
- W' is the group (c):



in which Z' is the oxygen of the phenolic hydroxyls involved in the coupling, Y' is -CO-, E' is the inert spacing structure -CH₂-CH₂- and n is zero.

Particular preference is given to the immunotoxins formed by one or more structures containing trichosanthin or trichokirin and an antibody P, which are represented by the statistical formula:



$$P'(W'-A')_m \quad (III)$$

in which P', W' and A' are as defined above and m represents the number of groups belonging to the protein P which are involved in the coupling. The number m
5 varies from 0.3 to 12, preferably from 0.5 to 10.

The expression "m varies from 0.3 to 12, preferably from 0.5 to 10" means that the value of m is a statistical value because the coupling does not take place homogeneously within the population of
10 antibody molecules. The number m may therefore not be an integer.

The value of m depends especially on the antibodies used and more particularly on their molecular weight.

15 Thus, if a fragment Fab or Fab' is used as the starting antibody P, the value of m can vary between 0.3 and about 2; if a fragment F(ab')₂ is used, m can vary between 0.5 and about 4; for an antibody of the IgG type, the value of m will be between 0.5 and 6;
20 finally, for an antibody IgM, the value of m can vary between 1 and 12.

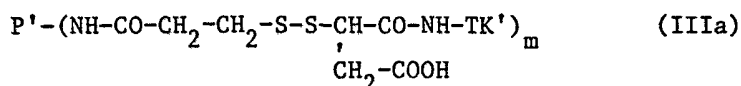
It is preferable, however, for the degree of substitution on the antibody P to be such as to lead to a value of m which is not less than 0.5 and not more
25 than 10.

More generally, the structures (I) and (II) above represent statistical formulae written in simplified form, as already explained.

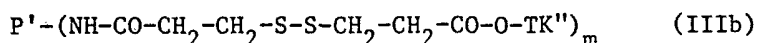
Analogously, the formulae (IV), (V) and (VI)
30 below are also statistical formulae - whenever n is 1 - because the coupling reactants are prepared from populations of proteins P₁ and P₂ which all have exactly the same properties as those taken into account above for the antibody P, whether these proteins P₁ and P₂
35 themselves are the antibody P or the protein extracted

from *Trichosanthes kirilowii*.

The immunotoxins having the statistical formula :



5 wherein P' and m are as above defined and TK' is the radical of the trichokirine as such, from which amino functions have been removed, and the immunotoxins having the statistical formula :



10 wherein P' and m are as herein above defined and TK'' is the radical of the trichokirine as such, from which phenolic hydroxy functions have been removed, are particularly actives.

According to another feature, the present invention relates to a process for the preparation of an immunotoxin having the structure (I) above, wherein a protein P₁, which
15 is arbitrarily either optionally modified trichosanthin or trichokirin or an antibody or antibody fragment, carrying at least one free thiol group attached to the said protein P₁ directly or via a spacing structure, is reacted, in aqueous solution and at room temperature, with a protein P₂
20 different from P₁, which is arbitrarily either optionally modified trichosanthin or trichokirin or an antibody or antibody fragment, carrying a group capable of coupling with the free thiol of the protein P₁ to form a thioether or disulfide bond.

25 According to a preferred feature, the present invention relates to a process for the preparation of an immunotoxin having the structure (II), in which P', W' and A' are as defined above, wherein a protein of the formula :

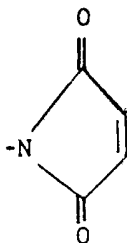


30 is reacted, in aqueous solution and at room temperature, with a protein of the statistical formula :



35 in which P₁' and P₂' represent the radicals of the proteins P₁ and P₂ bonded to the groups belonging to the said proteins or the radicals of protein P₁ or P₂ originating from the opening of the hydrate structures of the antibodies or antibody fragments or optionally of the protein A by reaction with periodic acid, Z, Z', Y, Y', E and

E' are as defined above and G represents a group :



5 or a group -S-S-X, in which X is an activating group.

Therefore, both P and A are proteins which arbitrarily have:

- (1) the thiol group or groups taking part in the coupling, and
- 10 (2) one or more functional groups capable of reacting with the above thiol groups to form a disulfide or thioether bond.

According to the present invention, the said thiol groups and functional groups are those of the
15 native proteins P or A or alternatively are introduced therein artificially.

For reasons of clarity, there now follows a description of the meaning of the symbols used to denote the above proteins or their radicals and of the
20 expressions used to denote the various symbols.

The symbol P represents a protein chosen from any antibody or antibody fragment, any immunoglobulin or immunoglobulin fragment, or any molecule derived from these molecules by artificial modification of any one of
25 their functional groups, including carbohydrate structures which they carry, with the proviso that the chosen protein is still capable of selectively recognizing a given antigen on the surface of the cells carrying this antigen, especially cancer cells.

30 The symbol A represents a protein which is the trichosanthin or the trichokirin, such as can be obtained directly from *Trichosanthes kirilowii*, or any molecule derived from these proteins by artificial modifica-

tion of any functional group carried by these proteins,
with the proviso that the chosen protein still has the
property of inhibiting ribosomal protein synthesis in
the eucaryotic cells, as can be demonstrated in an acel-
5 lular study model.

The symbol P' represents a radical derived from
the above protein P , as such or appropriately chemically
modified, from which one or more of its own groups have
been removed and in which other functional groups are
10 optionally blocked.

The symbol A' represents a radical derived from
the above protein A , as such or appropriately chemically
modified, from which one or more of its own groups have
been removed and in which other functional groups are
15 optionally blocked.

The symbol P_1 represents one of the proteins A
and P as defined above, which carries free thiol groups
attached to the said protein directly or via a spacing
structure.

20 The symbol P_2 , which is different from P_1 ,
represents one of the proteins A and P as defined above,
which carries one or more functional groups capable of
reacting with the free thiols.

The symbol P_1' represents that radical of the
25 protein P_1 which bears the groups belonging to
the protein P_1 , especially the groups SH (of the
cysteine), NH_2 (in the terminal position of the protein
or in the epsilon position of the lysines), OH (of the
tyrosines) or COOH (of the aspartic and glutamic acids),
30 or that radical of the protein P_1 which originates from
the opening of the carbohydrate structures by reaction
with periodic acid, when P_1 denotes an antibody or
antibody fragment or optionally the protein A .

35 The symbol P_2' represents that radical of the
protein P_2 which bears the characteristic functional

groups NH_2 (in the terminal position of the protein or in the epsilon position of the lysines), OH (of the tyrosines or COOH (of the aspartic and glutamic acids).

For example, $\text{P}_1\text{'-SH}$ represents the protein P_1
5 (which can arbitrarily be the antibody or antibody fragment P or trichosanthin or trichokirin) in which the SH groups of the cysteines are free and the other functional groups are optionally blocked.

In the same way, $\text{P}_1\text{'-CO-}$ represents the protein
10 P_1 in which the terminal carboxyl group or the carboxyl groups of its glutamic and aspartic acids are coupled with a group which artificially introduces an SH group.

Again, $\text{P}_2\text{'-NH-}$ represents the protein P_2 (which
15 can arbitrarily be the antibody or antibody fragment P or trichosanthin or trichokirin) in which the terminal amino group or the amino groups of its lysines are attached to a group capable of coupling with the thiol of the protein P_1 .

The term "inert spacing structure", as used
20 here for E and E', denotes a divalent organic radical which is inert towards the reactants used in the process, such as a straight-chain or branched alkylene group containing from 1 to 15 carbon atoms, which can contain one or more double bonds, can be interrupted by oxygen
25 atoms or can be substituted by one or more inert functional groups such as methoxy groups, free or esterified carboxyl groups, dialkylamino groups or carbamate groups. The same term also denotes an arylene
30 group containing from 6 to 15 carbon atoms, which can be substituted by one or more inert functional groups as indicated above for the alkylene group.

The expression "functional group capable of
bonding covalently", as used here for Y and Y', denotes
any groups capable of reacting with the groups belonging
35 to the proteins P_1 and P_2 to give a covalent bond. Thus,

for example the groups -CO- are suitable functional groups capable of bonding with the free amines, the thiols and the phenolic hydroxyls of the proteins. Likewise, the group -NH- is a suitable functional group capable of bonding with the free carboxyl groups of the proteins. The group =N- is a suitable functional group capable of bonding with the two carbon atoms of the carbohydrate structures of the proteins P₁ or P₂ after oxidation with periodate ions, when P₁ or P₂ represents an antibody or antibody fragment or optionally the protein A.

The expression "group belonging to the proteins", as used here for Z, Z' and Z", denotes the radicals originating from the characteristic groups of the amino acids forming the proteins P₁ and P₂, such as the oxygen atom originating from the hydroxyls of the tyrosine and possibly serine amino acids, the carbonyl group originating from the terminal carboxyl or the free carboxyls of the aspartic and glutamic acids, the -NH- group originating from the terminal amine of the proteins or the lysines, or the sulfur atom originating from the thiol of the cysteine. The same expression also denotes the group originating from the dialdehyde structure obtained after oxidation of one of the carbohydrate structures of the proteins P₁ or P₂ by treatment with periodate ions, when P₁ or P₂ represents an antibody or antibody fragment or optionally the protein A.

The term "activating radical", as used here for X, denotes a group bonded to an -S-S- bridge and capable of reacting with a free thiol to form a disulfide with the release of X-SH. Suitable activating radicals are pyridin-2-yl and pyridin-4-yl groups which are unsubstituted or substituted by one or more halogens or alkyl, carboxyl or alkoxy carbonyl radicals; the phenyl group which is unsubstituted or, preferably, substituted by one or more halogens or nitro, alkoxy,

carboxyl or alkoxycarbonyl groups; or an alkoxycarbonyl group such as methoxycarbonyl.

The terms "alkyl" and "alkoxy" denote groups containing up to 5 carbon atoms.

5 The term "alkylene" denotes straight-chain or branched saturated aliphatic groups containing up to 10 carbon atoms, which can be substituted by one or more inert functional groups such as alkoxycarbonyl groups.

10 Trichosanthin is a ribosome-inactivating protein (RIP) extracted from the roots of *Trichosanthes kirilowii* and described in Nature 195 (1,2), 321 (1986). Extraction of the seeds of *Trichosanthes kirilowii* gives a novel and exceptionally powerful RIP which is different from trichosanthin. This novel RIP, called "trichokirin",
15 has the following characteristics:

- it is a glycoprotein with a molecular weight of 28,000 \pm 3,000 as determined by electrophoresis on polyacrylamide gel in the presence of SDS (sodium dodecyl-sulfate);
- 20 - it has an isoelectric point \geq 9;
- it has a content of neutral sugars of 1.1 to 1.5% by weight, including 0.3 to 1.2% of mannose;
- it has the following amino acid composition, expressed
25 as the number of residues per mol of protein \pm 20%:

Lys : 17.3; His : 1.1; Arg : 6.7; Asx : 22.8;
Thr : 18.9; Ser : 23.5; Glx : 21.6; Pro : 8.0;
Gly : 16.0; Ala : 21.1; 1/2 Cys: 1.9;
Val : 12.5; Met : 3.05; Ile : 15.8; Leu : 24.3;
30 Tyr : 12.1; Phe : 10.1; Trp : n.d.

where Asx is the aspartic acid and asparagine residues together, Glx is the glutamic acid and glutamine residues together, 1/2 Cys is the cysteine residue of the native protein under the form of cysteic acid determined during the analysis

methyl cellulose. Elution can be carried out using a linear concentration gradient, for example a 0 to 0.3 M aqueous solution of sodium chloride. Normally, 4 main peaks are eluted and peak IV, eluted by a sodium chloride concentration of 0.03 to 0.11 M, contains the major part of the trichokirin.

The trichokirin can be purified by gel filtration, for example on a column of Sephadex (registered trademark).

Trichokirin can be coupled to antibodies or antibody fragments via an appropriate coupling agent by the process described above.

Trichokirin inhibits protein syntheses in a lysate of reticulocytes with an ID_{50} of 1.5-4 ng/ml. The effect is very much less on whole cells, the ID_{50} varying from 7 to more than 100 $\mu\text{g/ml}$ according to the cell examined.

Table 1 shows the effect of trichokirin on a variety of cells, especially the ID_{50} (in $\mu\text{g/ml}$ and $M \cdot 10^{-7}$) for the inhibition of protein synthesis; this was determined by applying the method described in J. Biol. Chem. 259, 9359-9364 (1984).

TABLE 1

Cell	ID_{50} $\mu\text{g/ml}$	$M \cdot 10^{-7}$
Human fibroblasts	3.9	1.3
TG cells	7.5	2.5
NB 100 cells (neuroblastoma)	10.3	3.4
JAR cells (choriocarcinoma)	16.3	5.4
HeLa cells	45.0	15.0
M 4039 (melanoma)	9.9	3.3

The preparation of monoclonal antibodies directed against human cancer cells has been widely mentioned in

the scientific literature and many of these antibodies are now commercially available.

The chemical coupling of trichosanthin or trichokirin with the antibody (or antibody fragment) can be effected by the process of the present invention using procedures which:

- preserve the respective biological activities of the two components of the conjugate, namely the antibody and the trichosanthin or trichokirin,
- ensure that the process has a satisfactory reproducibility and a good coupling yield,
- make it possible to control the value of the toxin-to-antibody ratio in the conjugate obtained, and
- lead to a stable and water-soluble product.

Among the procedures corresponding to these characteristics, preference must be given to those which involve one or more thiol groups for forming the bond between the 2 proteins. In fact, these thiol groups are particularly suitable for forming either disulfide bonds or thioether bonds, both of which satisfy the general conditions above. These procedures are disclosed in US patent 4340535 and in European patent 169111.

In general, in order to carry out the coupling reactions between proteins successfully and to eliminate disordered crosslinkings in particular, it is important for one of the proteins to be coupled, and one only, to carry the thiol or thiol groups to be used, while only the other protein carries one or more groups capable of reacting with the thiols in an aqueous medium having a pH of between 5 and 9, and at a temperature not exceeding 30°C, to produce a stable and clearly defined covalent bond.

The characteristics of the proteins P_1 and P_2 used as starting materials are illustrated in detail below. The spacing structure E can be replaced by the preferred structures R to R_8 , which are only given as

examples.

THE PROTEIN P_1

As this protein is in all cases the one carrying the thiol group or groups which will take part in the coupling, the situation which arises varies according to the nature of this protein P_1 .

A) The protein P_1 carries, in the natural state, one or more thiol radicals which can be used to permit coupling with the protein P_2 ; this is particularly the case if the protein P_1 is the antibody fragment known as $F(ab)'$, as conventionally obtained by the limited proteolysis of the antibody in the presence of pepsin followed by reduction of the disulfide bridge (or bridges) between high-molecular chains.

This is also the case if the protein P_1 is trichosanthin or trichokirin or a derivative of one of these toxins in which at least one of the thiol groups carried by the cysteine residues of the native toxin is free and accessible for chemical coupling.

In all cases, the protein P_1 carrying its natural thiol group (or groups) can be used in this state for the coupling step.

B) The protein P_1 does not carry, in the natural state, thiol radicals which can be used to permit coupling with the protein P_2 :

- this is especially the case if the protein P_1 is a native immunoglobulin, a whole antibody or an antibody fragment, especially one of the fragments commonly called $F(ab)_2'$ or $F(ab)$;
- another case in which the protein P_1 does not carry, in the natural state, a thiol group which can be used for coupling is the case where this protein P_1 is trichosanthin or trichokirin in which each of the cysteine residues is either blocked by alkylation or inaccessible for chemical modification.

In all cases, it will then be appropriate artificially to introduce into such molecules one or more thiol groups capable of permitting coupling.

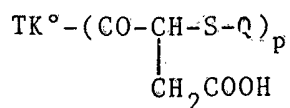
5 Three types of reaction can preferably be used for the introduction of thiol groups:

1 - The first type of reaction is with S-acetylmercaptosuccinic anhydride, which is capable of acylating amine groups of the protein. It will then be possible to free the thiol groups by reaction with hydroxylamine to
10 remove the acetyl protecting radical, in the manner already described (Archives of Biochemistry and Biophysics, 119, 41-49, (1967)). It will even be possible, in the case where the thiol group (or groups) thus introduced in the protected form are subsequently to
15 react with an activated mixed disulfide radical, to dispense with the prior deprotection by means of hydroxylamine; in fact, the reaction for formation of the disulfide bond using the reactants forming the subject of the present invention takes place just as
20 well with the S-acetyl radical as with the free thiol.

Other methods described in the scientific literature can also be used to introduce thiol groups into the protein to be modified.

25 One particularly advantageous modified protein P_1 is a S-acetylmercaptosuccinoyl-trichokirin obtained by treating trichokirin with S-acetylmercaptosuccinic anhydride, another being the corresponding mercaptosuccinoyl derivative prepared by reacting hydroxylamine with the S-acetyl derivative.

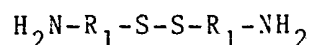
30 This modified trichokirin can be represented by the formula:



in which TK° represents the trichokirin radical, Q

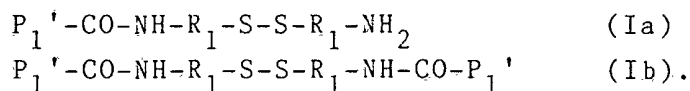
represents hydrogen or the acetyl group and p can vary between 0.2 and 3, preferably between 0.4 and 1.

2 - The second type of reaction consists in reacting the protein via its carboxyl groups with a symmetrical diamine molecule having a disulfide bridge, of the formula:



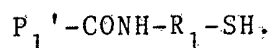
in which R_1 is an aliphatic group containing from 2 to 5 carbon atoms.

10 The reaction is preferably carried out with cystamine [$\text{R}_1 = -(\text{CH}_2)_2-$] in the presence of a coupling agent such as a carbodiimide and especially a water-soluble derivative like 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide, and leads to the formation, depending on the stoichiometries used, of one of the following derivatives or a mixture of both:



20 A reaction product of this type can then be used in two ways:

a) If, in the formulae Ia and Ib, the radical P_1' is the radical of protein P_1 which is trichosanthin or trichokirin or one of their derivatives, the reaction medium obtained is subjected, without fractionation, to the action of a reducing agent such as 2-mercaptoethanol, which gives a single protein derivative of the general formula:



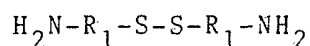
The product thus obtained is then purified by dialysis or gel filtration.

30 b) If, in the formulae Ia and Ib, the radical P_1' is that radical of the protein P_1 which consists of

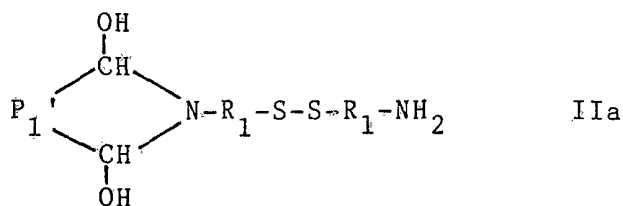
an antibody or one of its fragments, the reaction medium obtained will be used as such for the coupling, in which case a thiol/disulfide exchange method will be used, for example the one described by Gilliland and Collier

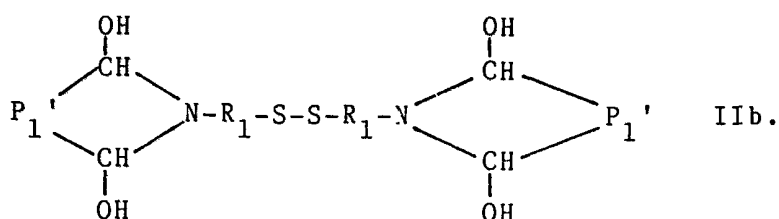
5 (Cancer Research, 40, 3564, (1980)).

3 - The third type of reaction consists in using carbohydrate units, which are present in the natural state in the antibodies, in order to attach the radical carrying the thiol which it is proposed to introduce. The protein is then subjected to oxidation with periodate ions in order to create aldehyde groups on the carbohydrate units. The reaction is stopped by the addition of a reagent which consumes the remaining periodate, for example an excess of ethylene glycol, and the by-products are removed by dialysis or any other appropriate treatment. The product obtained is treated with a symmetrical diamine molecule having a disulfide bridge, of the general formula:



20 in which R_1 is an aliphatic group containing from 2 to 5 carbon atoms. The addition products formed are then reduced to secondary or tertiary amines by reaction with a suitable metal hydride, especially sodium borohydride. The reaction is preferably carried out with cystamine [25 $\text{R}_1 = -(\text{CH}_2)_2-$] and leads to the formation, depending on the stoichiometries used, of one of the following derivatives or a mixture of both:

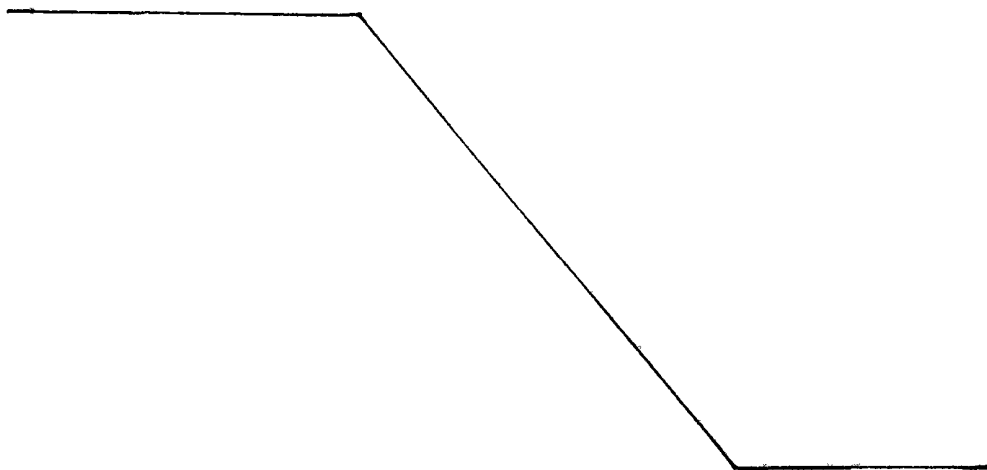




5 The reaction medium may then be treated exactly as indicated above for the products characterized by the structures Ia or Ib in which P₁' represents an antibody or antibody fragment.

10 In the last two types of reaction, described above, for the artificial introduction of thiol groups (the types using a symmetrical diamine disulfide reactant), the protein P₁ used preferably possesses no free SH groups.

15 In the case of antibodies or antibody fragments and, more generally, all the substances of the first group, as defined previously, which do not possess naturally free SH groups, it will be appropriate to carry out a reductive methylation, for example by the method of MEANS and FEENEY; in this way, it is usually possible to introduce several dozen methyl radicals per



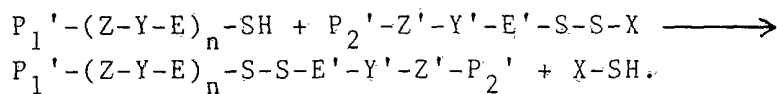
mol. of antibody without modifying its capacity to selectively recognize an antigen on the surface of the cells carrying this antigen.

THE PROTEIN P₂

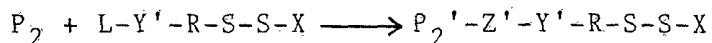
5 This protein is in all cases the one which carries one or more functional groups capable of reacting with the thiols of the protein P₁ to form either a disulfide or a thioether bond. These functional groups, which are always introduced artificially into
10 the protein P₂, differ according to whether it is desired to effect coupling by a disulfide bond or by a thioether bond and are chosen as indicated below.

A) The disulfide bond

15 In this case, the preparation of the conjugate can be represented by the equation:



20 The protein P₂' substituted by an activated sulfur atom is obtained from the protein P₂ itself or from the correctly protected protein P₂ by substitution with the aid of a reagent which itself carries an activated sulfur atom, according to the equation:



in which:

- 25 - P₂ denotes the protein to be substituted and
- L-Y' represents a group permitting the covalent attachment of the reagent to the protein.

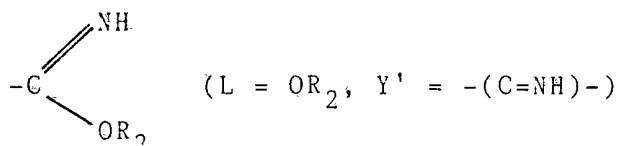
30 The functional group L-Y' is a group capable of bonding covalently with any one of the groups carried by the side chains of the constituent amino acids of the protein to be substituted. Among these groups, the following will be singled out in particular:

- a) the amine end groups of the peptide chains or the amine side groups of the lysyl radicals contained

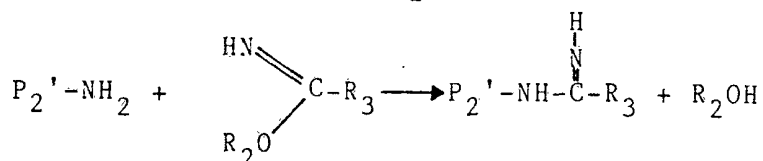
in the protein.

In this case, L-Y' can represent especially:

- a carboxyl group which can bond to the amine groups of the protein in the presence of a coupling agent such as a carbodiimide and especially a water-soluble derivative like 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide;
- a carboxylic acid chloride which is capable of reacting directly with the amine groups to acylate them;
- 10 - a so-called "activated" ester such as an ortho- or para-nitrophenyl or -dinitrophenyl ester, or alternatively an N-hydroxysuccinimide ester, which can react directly with the amine groups to acylate them;
- an internal anhydride of a dicarboxylic acid, for example succinic anhydride, which reacts spontaneously
- 15 with the amine groups to create amide bonds; or
- an imidoester group:



20 in which R₂ is an alkyl group, which reacts with the amine groups of the protein P₂ according to the equation:



in which R₃ represents the group -R-S-SX;

b) the phenol groups of the tyrosyl radicals contained in the protein.

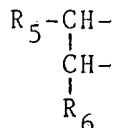
25 In this case, L-Y' can represent especially an imidazol-1-ylcarbonyl group, which reacts with the phenol groups of the protein according to the equation:



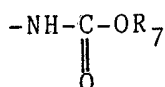
in which the imidazol-1-yl is L, the CO group is Y' and R₄ is the group -R-S-S-X.

5 The radical -S-S-X denotes an activated mixed disulfide capable of reacting with a free thiol radical. In particular, in this mixed disulfide, X can denote a pyridin-2-yl or pyridin-4-yl group optionally substituted by one or more alkyl, halogen or carboxyl radicals. X can also denote a phenyl group preferably substituted
10 by one or more nitro or carboxyl groups. Alternatively, X can represent an alkoxycarbonyl group such as the methoxycarbonyl group.

The radical R denotes the spacing structure (indicated as E in the general formula II above) capable
15 of carrying the substituents Y' and S-S-H simultaneously. It must be chosen so as not to contain groups capable of interfering, during the subsequent reactions, with the reactants used and the products synthesized. In particular, the group R can be a group -(CH₂)_n-, n being
20 between 1 and 10, or alternatively a group:



in which R₆ denotes hydrogen or an alkyl group having from 1 to 8 carbon atoms and R₅ denotes a substituent which is inert towards the reactants to be used sub-
25 sequently, such as a carbamate group:



in which R₇ denotes a linear or branched alkyl group

having from 1 to 5 carbon atoms, especially the tert.-butyl group.

The reaction of the compound L-Y'-R-S-S-X with the protein P₂ is carried out in a homogeneous liquid phase, most commonly in water or a buffer solution. If necessitated by the solubility of the reactants, a water-miscible organic solvent can be added to the reaction medium at a final concentration which can reach 20% by volume in the case of a tertiary alcohol, such as tertiary butanol, or 10% by volume in the case of dimethylformamide or tetrahydrofuran.

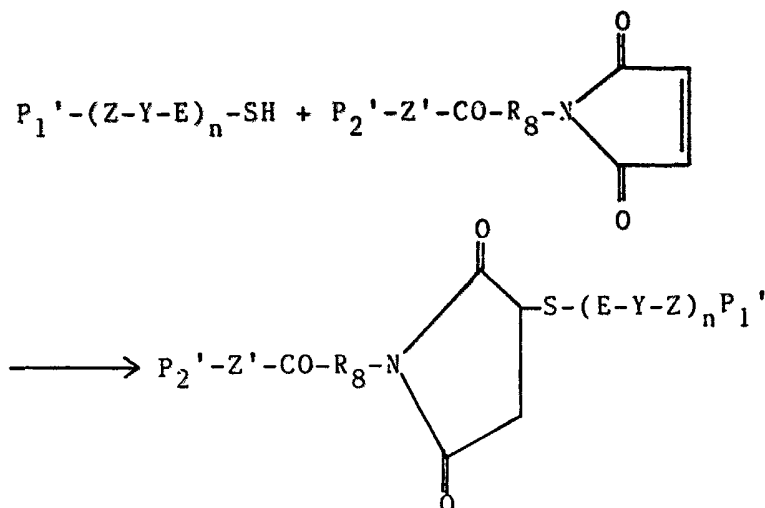
The reaction is carried out at room temperature for a time varying from a few minutes to a few hours, after which the low-molecular products, and in particular the excess reactants, can be removed by dialysis or gel filtration. This process usually makes it possible to introduce between 1 and 15 substituent groups per mol of protein.

When using such compounds, the coupling with the protein P₁ is carried out by bringing the two proteins together in an aqueous solution having a pH of between 6 and 8, at a temperature not exceeding 30°C, for a time varying from 1 hour to 24 hours. The aqueous solution obtained is dialyzed, if appropriate, to remove the low-molecular products, and the conjugate can then be purified by a variety of known methods.

B) The thioether bond

In this case, the preparation of the conjugate consists in reacting P₁'-(Z-Y-E)_n-SH with the protein P₂ into which one or more maleimide radicals have been introduced beforehand.

The reaction is then represented by the following equation, which is given as an example:



in which:

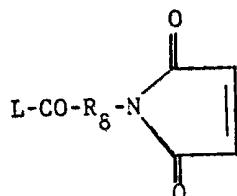
- 5 - R_8 represents an aliphatic or aromatic spacing structure containing from 1 to 15 carbon atoms, which is inert towards the reactants to be used subsequently, and
- 10 - Z' represents groups which can vary according to the type of functional group of the protein P_2 involved in the coupling. Thus, $Z' = \text{oxygen}$ in the case of an ester on the phenol of a tyrosyl residue; $Z' = \text{NH}$ in the case of the coupling of an activated carboxyl group with an amine group of the protein; or $Z' = \text{NH-CH}_2$ in the case of the reaction of a chloromethyl
- 15 ketone with an amine group of the protein.

20 The protein P_2 substituted by the maleimide group or groups is obtained from the protein P_2 itself, or the correctly protected protein P_2 , by substitution of suitable groups of the protein with the aid of a reagent which itself carries the maleimide group.

Among these suitable groups, the following will be singled out in particular:

- 25 a) the amine end groups of the peptide chains or the amine side groups of the lysyl residues contained in the protein. In this case, the reagent carrying the maleimide radical can be:

- either a reagent of the general formula:

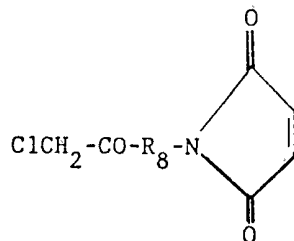


in which L-CO- represents:

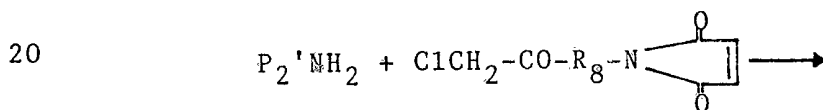
- either a carboxyl group, in which case the reaction is carried out, after activation of the carboxyl group, in the presence of a coupling agent such as a carbodiimide and especially a water-soluble derivative such as 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide,
- or a so-called "activated" ester such as an ortho- or para-nitrophenyl or -dinitrophenyl ester, or alternatively an N-hydroxysuccinimide ester, which reacts directly with the amine groups to acylate them.

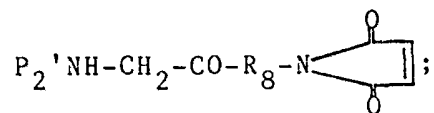
The preparation of such reagents is described especially in Helvetica Chimica Acta 58, 531-541 (1975). Other reagents in the same class are commercially available.

- or a reagent of the general formula:

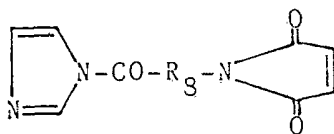


which is capable of reacting with the amine groups of the protein P₂ according to the equation:

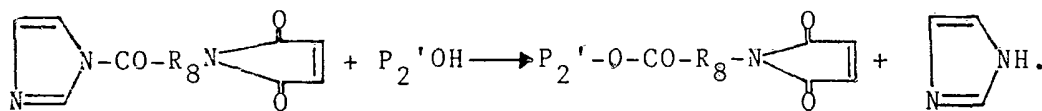




5 b) the phenol groups of the tyrosyl radicals contained in the protein. In this case, the reagent carrying the maleimide radical can be a reagent of the general formula:



which reacts with the phenol groups of the protein according to the equation:

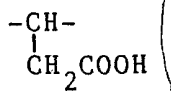


The reaction of the maleimide-carrying reagents with the protein P₂ is carried out in a homogeneous liquid phase, most commonly in water or a buffer solution. If necessitated by the solubility of the reactants, a water-miscible organic solvent can be added to the reaction medium at a final concentration which can reach 20% by volume in the case of a tertiary alcohol, such as tertiary butanol, or 10% by volume in the case of dimethylformamide or tetrahydrofuran.

5
10 The reaction is carried out at room temperature for a time varying from a few minutes to a few hours, after which the low-molecular products, and in particular the excess reactants, can be removed by dialysis or gel filtration. This process usually makes it possible to
15 introduce between 1 and 15 substituent groups per mol of protein.

When using such compounds, the coupling with the protein P₁ is carried out by bringing the two protein derivatives together in an aqueous solution having a pH of between 6 and 8, at a temperature not exceeding 30°C, for a time varying from 1 hour to 24 hours. The solution obtained is dialyzed, if appropriate, to remove the low-molecular products, and the conjugate can then be purified by a variety of known methods.

20
25 Among the compounds of the present invention, those which are particularly suitable are the compounds of the formula (II) in which W' represents one of the groups (a), (b), (c) and (d) above in which E and E' represent a group $-(CH_2)_p-$, p being an integer from 2 to 7, or a group:



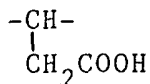
Thus, particularly valuable protein derivatives are represented by the statistical formula:



in which:

- 5 - P_2'' represents the radical of a protein selected from the group comprising:
- any antibody or antibody fragment, any immunoglobulin or immunoglobulin fragment or any molecule derived from these molecules by artificial modification of any one of their functional groups;
 - 10 and
 - trichosanthin or trichokirin or any molecule derived from the said proteins by artificial modification of any one of their functional groups,
- 15 the said radical being the protein from which one or more phenolic hydroxyl groups of the tyrosines have been removed;
- the oxygen atom is that belonging to the phenolic hydroxyl groups missing from the radical P_2'' ; and
 - 20 - E and G are as defined above.

Particular preference is given to the compounds of the formula (VI) in which E represents a group $-(CH_2)_p-$, in which p is an integer from 2 to 7, or a group:



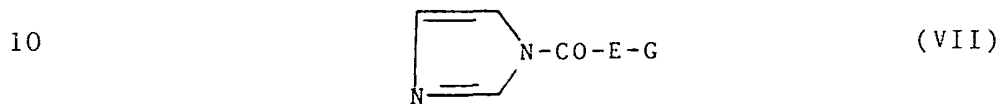
- 25 and G is a group of the structure $-S-S-X$, in which X is an activating radical chosen from the pyridin-2-yl and pyridin-4-yl groups which are unsubstituted or substituted by one or more halogens or alkyl, carboxyl or alkoxy-carbonyl radicals, the phenyl group which is unsubstituted or substituted by one or more halogens or
- 30

nitro, alkoxy, carboxyl or alkoxy-carbonyl groups, or an alkoxy-carbonyl group.

The products of the formula (VI) are prepared by reacting a product of the formula:



in which P_2'' is as defined above and the hydroxyl group is the phenolic hydroxyl missing from the tyrosines of the radical P_2'' , with a compound of the formula (VII) below:



at a temperature of 10 to 40°C, in an aqueous solvent optionally containing a water-miscible organic solvent, for example an ether solvent like dioxane or tetrahydrofuran.

15 The examples which follow provide a clearer understanding of the invention without limiting its scope.

The monoclonal antibody AT15E is directed against the antigen Thy 1.2 of murine lymphocytes. This antibody is the one described in Journal of Immunology 122, 2491-8 (1979) and has been obtained from the hybridoma described in Hybridoma 1(1), 13-17 (1981).

In the examples, trichokirin is denoted by the abbreviation TK.

25 **EXAMPLE 1: ISOLATION OF TRICHOKIRIN**

A) Crude extract

Seeds of *Trichosanthes kirilowii* (1.2 kg) are ground in an Ultraturrax apparatus with 8 volumes of a

0.14 M solution of sodium chloride containing 5 mM phosphate buffer, pH 7.5. Extraction is continued overnight at 4°C, with magnetic stirring.

5 After removal of the coarse residues, the extract is centrifuged at 10,000 g for 45 minutes at 0°C. The supernatant is decanted in the cold to remove the solidified fats, after which the proteins are precipitated by the addition of ammonium sulfate up to saturation point. After centrifugation as indicated
10 above, the precipitated product is resuspended in 1 liter of a 0.14 M aqueous solution of sodium chloride containing 5 mM phosphate buffer, pH 7.5. The undissolved product is removed by centrifugation under the same conditions as above. The supernatant is transferred to
15 a column of Sephadex G 25 coarse (95 cm x 10 cm), equilibrated with 5 mM phosphate buffer, pH 7.0, at room temperature, and eluted at a rate of 3 l/hour. The first peak is collected.

B) Ion exchange chromatography

20 The combined extracts from 2 operations (originating from 2.4 kg of seeds) are treated with sodium chloride up to a final concentration of 30 mM and transferred to a column of CM-Sepharose Fast Flow (17.5 cm x 5 cm), equilibrated with 30 mM phosphate
25 buffer, pH 7.0. The non-fixed product is eluted with the same buffer. The bound protein is eluted by a gradient containing from 30 to 110 mmol/l of sodium chloride in the same phosphate buffer (volume 20 liters).
30 400 ml fractions are collected at a rate of 1.2 liters per hour. The absorbance at 280 nm and the conductivity of the eluate are monitored. The fractions showing an inhibitory activity against protein synthesis are combined (3.6 l) and acidified to pH 5.8 with acetic acid. The solution is transferred to a column of S-Sepharose
35 Fast Flow (6.6 cm x 5 cm), equilibrated with 10 mM

sodium acetate buffer, pH 4.5. After washing with the same buffer, the fixed protein is eluted with 20 mM phosphate buffer, pH 7.5, containing 0.5 M sodium chloride.

5 The fraction corresponding to the peak containing the protein (200 ml) is collected, dialyzed against 3 times 50 l of distilled water and then lyophilized to give 380 mg of trichokirin.

C) Gel filtration

10 The trichokirin obtained above is dissolved in 50 ml of a 0.14 M solution of sodium chloride. An insoluble material is removed by centrifugation at 20,000 g for 30 minutes. The supernatant is transferred to a column of Sephadex G 50 coarse (95 cm x 5 cm),
15 equilibrated with 5 mM phosphate buffer, pH 7.0, at 4°C. Elution is carried out at a rate of 60 ml/hour. The solution corresponding to the peak containing the protein is collected, dialyzed and lyophilized as indicated above.

20 This gives 267 mg of purified trichokirin.

EXAMPLE 2: S-ACETYLMERCAPTOSUCCINOYL-TRICHOKIRIN AND
MERCAPTOSUCCINOYL-TRICHOKIRIN

100 microliters of a solution of S-acetyl-
mercaptosuccinic anhydride (SAMSA) containing 10.5 mg/ml
25 (60.33 micromol/ml) in dimethylformamide are added to a solution of 5.8 mg of trichokirin (0.215 micromol) in 6 ml of 125 mM phosphate buffer, pH 7 (i.e. 0.973 mg/ml, about 0.036 micromol/ml). Incubation takes one hour, after which the reaction medium is purified by dialysis
30 against 125 mM phosphate buffer, pH 7, to remove the excess reagent. This gives 5.7 ml of a solution of S-acetylmercaptosuccinoyl-trichokirin containing 0.948 mg/ml. Reaction with hydroxylamine by ELLMAN's method (Methods in Enzymology 25, 457, (1972)) gives mercapto-

succinoyl-trichokirin containing 0.7 free SH group per mol of trichokirin (spectrophotometric analysis). When examined by polyacrylamide gradient electrophoresis, this modified protein shows a single band with a molecular weight of the order of $28,000 \pm 3000$, which is identical to that of the native protein.

EXAMPLE 3: CONJUGATE OBTAINED BY REACTING THE ANTIBODY AT15E (ANTI-THY 1.2) WITH MERCAPTOSUCCINOYL-TRICHOKIRIN

A) Preparation of the modified antibody AT15E

10 The antibody AT15E is a monoclonal antibody directed against the antigen Thy 1.2 of murine lymphocytes. This antibody is the one described in Journal of Immunology 122, 2491-8 (1979) and has been obtained from the hybridoma described in Hybridoma 1(1), 13-17
15 (1981).

25 microliters of a solution of N-succinimidin-3-yl 3-(pyridin-2-yl-disulfanyl)propionate containing 5.5 mg/ml in ethanol are added to 3.7 ml of a solution of antibody AT15E containing 3.55 mg/ml (i.e. 0.087 micromol) in 0.1 M borate buffer, pH 8.8.

Incubation takes 30 minutes and the excess reagent is then removed by dialysis against 125 mM phosphate buffer, pH 7. After dialysis, the protein solution is centrifuged to give 3.3 ml of a solution containing 3.4 mg/ml. By spectrophotometric analysis at 343 nm of the pyridine-2-thione released by exchange with 2-mercaptoethanol, it is found that the antibody obtained carries 3.2 activated mixed disulfide groups per mol of antibody.

30 B) Preparation of the immunotoxin (conjugate)

5 ml of the solution of modified trichokirin obtained above (i.e. 0.183 micromol) are added to 1.9 ml of the solution of activated antibody obtained above (i.e. 0.043 micromol) and the mixture is incubated for

20 hours at 25°C in the presence of 350 microliters of a molar solution of hydroxylamine hydrochloride. The solution is centrifuged and then purified by filtration on a column of Sephadex G200, the optical density of the effluent being measured at 280 nm. Combination of the fractions containing both the antibody and the TK gives 20 ml of a solution of immunotoxin containing 0.215 mg/ml (i.e. 4.3 mg). This solution contains 0.045 mg/ml of modified trichokirin coupled with the antibody.

The average degree of coupling in this preparation is 1.5 TK per mol of antibody.

The conjugate so obtained has the above formula (III a) wherein P' is the radical of antibody AT15E devoid of amino functions and \underline{m} is 1.5.

IMMUNOTOXIN ACTIVITY TESTS

One of the properties of trichokirin is to inhibit protein synthesis in eucaryotic cells. The tests performed are therefore tests for the inhibition of protein synthesis:

- either on an acellular model
- or on a cell model.

A) The acellular model

The in vitro protocol uses appropriately complemented subcellular fractions of rat liver capable of incorporating ^{14}C -phenylalanine in the presence of an artificial messenger RNA: polyuridylic acid.

The procedure employed for preparing the subcellular fractions and measuring the incorporation of ^{14}C -phenylalanine is an adaptation of the method described in Biochimica et Biophysica Acta 312, 608-615 (1973), using both a microsomal fraction and a cytosol fraction of the rat hepatocytes. The sample containing the trichokirin is introduced in the form of a solution appropriately diluted in a 50 mM tris-HCl buffer, pH 7.6, containing 0.2% of 2-mercaptoethanol and 15 micrograms/ml of bovine serum albumin. The count data are used to calculate, relative to a reference medium without

inhibitor, the percentage inhibition of the incorporation of ^{14}C -phenylalanine for each reaction medium.

The values obtained together make it possible to determine the concentration of trichokirin (or IC_{50}) which inhibits the incorporation of ^{14}C -phenylalanine by 50% under the experimental conditions. Thus, the IC_{50} of native trichokirin was found to be equal to $7 \cdot 10^{-11}$ M, while that of the modified trichokirin was found to be equal to $8-10 \cdot 10^{-11}$ mol/liter. Therefore, the modification does not cause a significant loss of activity of the trichokirin.

B) Specific cytotoxicity of the trichokirin immunotoxin on a cell model

The biological activity of trichokirin is to inhibit protein synthesis in eucaryotic cell systems. The technique employed uses a cell model in which the effect of the substances studied on the incorporation of ^{14}C -leucine into cancer cells in culture is measured. The cells used belong to the T2 cell line derived from a T murine leukemia expressing the surface antigen Thy 1.2. The cells are incubated in the presence of different concentrations of the substance to be studied, and then, when incubation has ended, the degree of incorporation of ^{14}C -leucine by the cells treated in this way is measured.

This measurement is carried out by a technique adapted from the procedure described in Journal of Biological Chemistry 249(II), 3557-3562, (1974), using the tracer ^{14}C -leucine to determine the degree of protein synthesis. The radioactivity incorporated is determined here on the whole cells isolated by filtration.

On the basis of these determinations, it is possible to draw the dose/effect curves, plotting, on the abscissa, the molar concentration of trichokirin in the substances studied, and, on the ordinate, the incor-

poration of ^{14}C -leucine expressed as a percentage of the incorporation by control cells in the absence of any substance affecting protein synthesis.

5 It is thus possible to determine, for each substance studied, the concentration which causes a 50% inhibition of the incorporation of ^{14}C -leucine, or "50% inhibitory concentration" (IC_{50}). Table 2 shows the IC_{50} values obtained in the same experiment with the trichokirin immunotoxin on the one hand and
10 uncoupled trichokirin on the other.

TABLE 2

Product	50% inhibitory concentration
Trichokirin IT	2×10^{-10} M
Trichokirin	2×10^{-6} M

It can be seen that the trichokirin IT has a very strong cytotoxic activity which is more than
15 10,000 times higher than that of uncoupled trichokirin.

EXAMPLE 4: CONJUGATE OBTAINED BY REACTING THE ANTIBODY AT15E (ANTI-THY 1.2) WITH TRICHOKIRIN 3-(PYRIDIN-2-YLDI-SULFANYL)PROPIONATE

A) Preparation of the modified antibody

20 50 microliters of a solution of S-acetylmercapto-succinic anhydride (SAMSA) containing 35 mg/ml in dimethylformamide are added to 20.1 mg of a solution of antibody AT15E containing 4.02 mg/ml (i.e. 0.134 micromol). The reaction medium is incubated for 2 hours 30
25 minutes at 10°C and then purified by dialysis against 125 mM phosphate buffer, pH 7, to remove the excess reagent.

This gives 4.5 ml of a solution containing 3.7 mg/ml.

200 microliters of a 1 M solution of hydroxylamine hydrochloride are added to 4.5 ml of this solution of antibody. After incubation for 2 hours at 25°C, the reaction medium is purified by dialysis against 125 mM phosphate buffer, pH 7, to remove the hydroxylamine.

By spectrophotometric analysis of the SH groups released by Ellman's method, it is found that the IgG obtained carries 3.8 SH groups per mol of antibody.

B) Preparation of the coupling reagent

The coupling reagent is the imidazolide derived from 3-(pyridin-2-yl)disulfanylpropionic acid, for which the method of preparation has already been described in European Patent Application no. 169111.

215 mg of 3-(pyridin-2-yl)disulfanylpropionic acid are dissolved in 0.5 ml of tetrahydrofuran. 203 mg of CDI (carbonyldiimidazole) are added to this solution. The mixture is stirred for 15 min at 25°C. An evolution of CO₂ gas is observed. The reaction medium is used directly and immediately without purification.

C) Modification of the trichokirin

18.5 microliters of the solution of the coupling reagent in THF are added to 5 mg of TK in 5 ml of 125 mM phosphate buffer, pH 7 (i.e. 0.185 micromol). The mixture is incubated for 15 minutes at 25°C and the reaction medium is then purified by dialysis against 125 mM phosphate buffer, pH 7, to remove the excess reagent. After centrifugation (15 min at 15,000 rpm), 4.4 ml of a solution of modified TK containing 0.94 mg/ml are obtained.

By spectrophotometric analysis at 343 nm of the pyridine-2-thione released by exchange with 2-mercaptoethanol, it is found that the TK obtained carries 0.6 activating group per mol of TK.

D) Preparation of the immunotoxin

1.8 ml of the solution of TK (i.e. 0.063 micromol) are added to 0.5 ml of the solution of antibody

obtained above (i.e. 0.012 micromol). The mixture is incubated for 20 hours at 30°C.

The reaction medium is purified by passage through an ACA44 column marketed by IBF-Rhone-Poulenc, elution being followed by measurement of the optical density of the effluent at 280 nm. Elution is carried out using PBS buffer (saline phosphate) to give 6.6 ml of a solution of immunotoxin containing 0.146 mg/ml (i.e. 0.966 µg). This solution contains 0.022 mg/ml of modified TK. The average degree of coupling of this preparation is one TK per mol of antibody.

The conjugate so obtained has the above formula (IIIb) wherein P' is the radical of antibody AT15E from which amino functions have been removed and m is 1.
E) Immunotoxin activity test.

The cytotoxic activity of the immunotoxin is measured by the same procedure as that described in Example 3.

Table 3 shows the IC₅₀ values obtained in the same experiment with, on the one hand, the trichokirin immunotoxin conjugated by the tyrosines, and, on the other hand, uncoupled trichokirin.

TABLE 3

Product	50% inhibitory concentration
Trichokirin IT	3×10^{-10} M
Trichokirin	3×10^{-6} M

It can be seen that the IT prepared by conjugating the anti-Thy 1.2 antibody with trichokirin by its tyrosine residues has a very strong cytotoxic activity which is 10,000 times higher than that of uncoupled trichokirin.

**EXAMPLE 5: CONJUGATE OBTAINED BY REACTING THE ANTI-THY
1.2 ANTIBODY AT15E SUBSTITUTED BY AN ACTIVATED
DISULFIDE GROUP WITH TK INTO WHICH A THIOL
HAS BEEN INTRODUCED**

5 A) Preparation of the modified TK

168 microliters of an aqueous solution of 3,3-
dimethyldithio-bis-propionimidate (DTBP) are added to
a solution of 7.25 mg of TK in 8.5 ml of 125 mM phosphate
buffer, pH 7 (i.e. 0.853 mg/ml). Incubation takes 1
10 hour at 28°C and the reaction medium is then purified
by dialysis against 125 mM phosphate buffer, pH 7, to
remove the excess reagent. The disulfide bridge of the
DTBP attached to the protein is then reduced with 2-
mercaptoethanol (at a final concentration of 1 per cent
15 for 1 hour at 30°C).

Dialysis against 125 mM phosphate buffer, pH
7, is then continued as before. After centrifugation,
7 ml of a solution of modified TK containing 0.794 mg/ml
are obtained. 0.6 free SH group per mol of protein can
20 be determined in this product by Ellman's method.

B) Preparation of the modified antibody

21 microliters of a solution containing 5.2 mg
of N-succinimidin-3-yl 3-(pyridin-2-yl-disulfanyl)pro-
pionate dissolved in 1 ml of ethanol are added to 3 ml
25 of a solution of antibody AT15E containing 4 mg/ml in
0.1 M borate buffer, pH 8.8. Incubation takes $\frac{1}{2}$ hour
at 28°C and the excess reagent is then removed by
dialysis against 125 mM phosphate buffer, pH 7. After
dialysis, the protein solution is centrifuged to give
30 2.3 ml of a solution containing 3 mg/ml.

By spectrophotometric analysis at 343 nm of the
pyridine-2-thione released by exchange with 2-mercapto-
ethanol, it is found that the antibody obtained carries
3.8 activated mixed disulfide groups per mol of antibody.

C) Preparation of the immunotoxin

5.4 ml of the solution of modified TK obtained in A) are added to 2 ml of the solution of modified antibody obtained above and the mixture is incubated for 6 hours at 28°C. The solution is centrifuged and then purified by filtration on a column of Sephacryl S200 HR, the optical density of the effluent being measured at 280 nm.

Combination of the fractions containing both the antibody and the TK gives 14 ml of a solution of immunotoxin containing 0.09 mg/ml. The average degree of coupling in this preparation is 1.5 mol of TK per mol of antibody.

The conjugate so obtained has the above formula (III) wherein :

- P' is the radical of antibody AT15E from which amino functions have been removed

- W' is the group $-Z'-Y'-E'-S-S(E-Y-Z)_n$ wherein

Z' is NH ;

Y' is CO ;

E' is $-CH_2-CH_2-$

E is $-CH_2-CH_2-$

Y is $-C=NH-$

n is 1

A' is the radical of the trichokirine from which the amino functions have been removed in is 1.5.

The activity of the immunotoxin is measured by the same procedure as that described in Example 3. Table 4 shows the IC_{50} values obtained in the same experiment with, on the one hand, the trichokirin immunotoxin prepared as above, and, on the other hand, uncoupled trichokirin.

TABLE 4

Product	50% inhibitory concentration
Trichokirine IT	$8-10 \times 10^{-11}$ M
Trichokirin	3×10^{-6} M

5

It can be seen that the IT has a very strong cytotoxic activity which is 30,000 times higher than that of uncoupled trichokirin.

10 This novel class of immunotoxins can be used to treat cancerous or non-cancerous diseases where the target cells to be destroyed would be recognized by the

antibody or antibody fragment used to prepare the immunotoxin. The optimum modes of administration and the duration of the treatment will have to be determined in each case according to the subject and the
15 nature of the disease to be treated.

The physiologically active products of the invention can be formulated for administration by any suitable method. Usually, the active principle will
20 be administered by injection and will be formulated in an apyrogenic sterile liquid, preferably water, which can contain physiologically acceptable salts, such as buffers or sodium chloride, so as to give an isotonic solution.

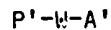
25 For an antitumoral treatment, the daily dose of trichosanthin or trichokirin as a conjugate is preferably between 1 and 100 mg and advantageously from 5 to 50 mg, for example about 10 mg.

30 The dosage units containing trichosanthin or trichokirin in the form of a conjugate, for example ampoules for injection, will therefore usually contain from 1 to 100 mg of trichokirin or trichosanthin conjugate.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:-

1. An immunotoxin formed by the coupling of an antibody with trichosanthin or trichokirin, having the following statistical formula:
as hereinbefore defined

05



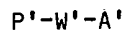
in which P' represents the radical of a protein P which is an antibody or antibody fragment, as such or appropriately chemically modified, from which one or more of its ~~own~~ ^{functional} groups have been removed and in which other functional groups are optionally blocked, A' represents the radical of a protein A which is trichosanthin or trichokirin, as such or appropriately chemically modified, from which one or more of its ~~own~~ ^{functional} groups have been removed and in which other functional groups are optionally blocked, and W represents a divalent covalent structure containing at least one thioether group or one disulfide group in which either one of the sulfur atoms is either selected among the ones of the cysteines of P or A or is bonded to the groups belonging to P and/or A by spacing structures carrying a functional group bonded to the said groups belonging to P and A.

20

2. An immunotoxin as claimed in claim 1, wherein A' is the radical of trichokirin, as such or appropriately chemically modified.

3. Immunotoxin formed by the coupling of an antibody with trichokirin or trichosanthin, having the following statistical formula :

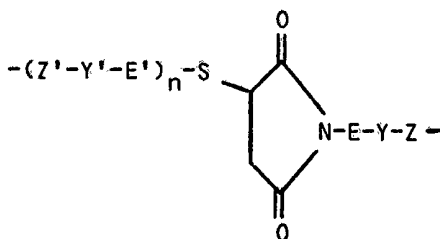
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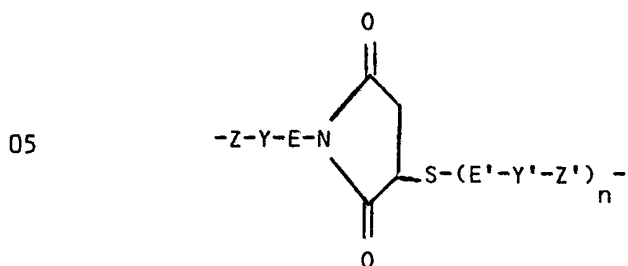
in which P' and A' are as defined in claim 1 and W' represents a covalent structure selected from the group consisting of :

30

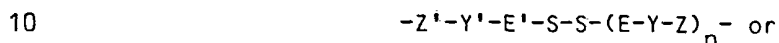
(a) a group of the formula :



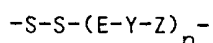
(b) a group of the formula :



(c) a group of the formula :



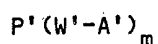
(d) a group of the formula :



15 in which :

- Z and Z', which are identical or different, represent the groups belonging to the proteins A and P, chosen from the oxygen atom originating from the hydroxyl of one of the tyrosine residues, the carbonyl group originating from one of the terminal carboxyls or the free carboxyls of the aspartic and/or glutamic acids of A and P, the group originating from the dialdehyde structure obtained after oxidation of the carbohydrate structure of P or optionally of the protein A with periodic acid, and the -NH- group originating from one of the terminal amines of A and P or from one of the amines in the epsilon position of one of the lysine residues ;
 - Y and Y' represent groups covalently bonded to any one of the groups Z and Z' of the proteins A and P ;
 - E and E' represent inert spacing structures ; and
 - n represents zero or 1.

30 4. An immunotoxin having the following statistical formula:

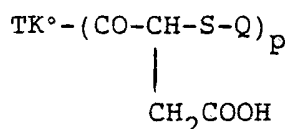


in which W' is as defined in claim 3, m varies from 0.3 to 12, P' represents the radical of a protein P which is an antibody or antibody fragment, as such or appropriately chemically modified,



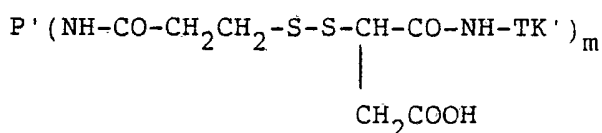
from which one or more of its functional groups have been removed and in which the other functional groups are optionally blocked, and A' represents the radical of a protein, as such or appropriately chemically modified, from which one or more of its functional groups have been removed and in which the other functional groups are optionally blocked.

5. Immunotoxin according to claim 1, wherein the protein A is the modified trichokirin represented by the formula:



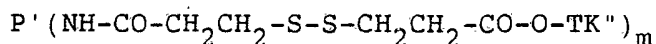
in which TK^o represents the trichokirin radical, Q represents hydrogen or the acetyl group and P can vary between 0.2 and 3.

6. An immunotoxin as claimed in claim 4, having the following statistical formula:



in which P' and m are as defined in claim 4 and TK' is the radical of trichokirin, as such, from which one amino function has been removed.

7. An immunotoxin as claimed in claim 4, having the following statistical formula:



in which P' and m are defined in claim 3 and TK'' is the radical of trichokirin, as such, from which one phenolic hydroxyl group has been removed.

8. A process for the preparation of an immunotoxin having the following statistical formula as hereinbefore defined:



in which P' represent the radical of a protein which is an antibody or antibody fragment, as such or appropriately chemically modified, from which one or more of its functional groups have been removed and in



which the other functional groups are optionally blocked, A' represents the radical of a protein A which is trichosanthin or trichokirin, as such or appropriately chemically modified, from which one or more of its ^{functional} ~~own~~ groups have been removed and in which

05 the other functional groups are optionally blocked, and W represents a divalent covalent structure containing at least one thioether group or one disulfide group in which either one of the sulfur atoms is either selected among the ones of the cysteines of P or A or it is bonded to the groups belonging to P and/or A by

10 spacing structures carrying a functional group bonded to the said groups belonging to P and/or A, wherein a protein P₁, which is arbitrarily either optionally modified trichosanthin or trichokirin or an antibody or antibody fragment, carrying at least one free thiol group attached to the said protein P₁ directly or via a

15 spacing structure, is reacted, in aqueous solution and at room temperature, with a protein P₂ different from P₁, which is arbitrarily either optionally modified trichosanthin or trichokirin or an antibody or antibody fragment, carrying a group capable of coupling with the free thiol of the protein P₁ to form a thioether or disulfide bond.

20

9. Process according to claim 8, wherein the protein derivative having the hereinbelow statistical formula ^{as hereinbefore defined} is used as protein P₁ or P₂ :



25

in which :

- P₂'' represents the radical of a protein selected from the group comprising :

30

- . any antibody or antibody fragment, any immunoglobulin or immunoglobulin fragment or any molecule derived from these molecules by artificial modification of any one of their functional groups ; and
- . trichosanthin or trichokirin or any molecule derived from the said toxins by artificial modification of any one of their functional groups,

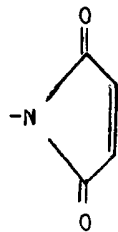


the said radical being the protein from which one or more phenolic hydroxyl groups of the tyrosines have been removed ;

- the oxygen atom is that belonging to the phenolic hydroxyl groups missing from the radical P₂"; and

05 - E is as defined in claim 3, and

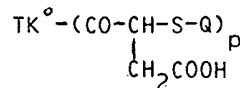
- G represents a group :



or a group -S-S-X wherein X is an

10 activating group.

10. Process according to claim 8, wherein the protein A is the modified trichokirine of formula :

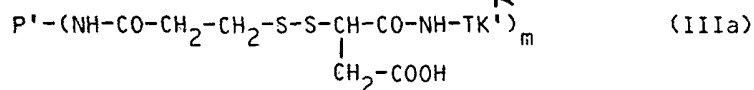


15

in which TK^o represents the trichokirin radical, Q represents hydrogen or the acetyl group and p can vary between 0.2 and 3.

11. Process according to claim 8, for the obtention of an immunotoxin having the statistical formula ^{as hereinbefore defined} :

20



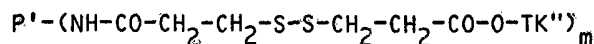
wherein :

25

- P' and m are as defined in claim 4 and TK' is the radical of trichokirine, as such, from which amino functions have been removed which consists in reacting, in an aqueous solution and at the ambient temperature, a protein P₁ which is an antibody or an antibody fragment bearing at least one free thiol group attached to the said protein P₁ directly or via a spacing structure, with the mercaptosuccinoyl-trichokirine as protein P₂.

30

12. Process according to claim 8 for the obtention of an immunotoxin having the statistical formula :



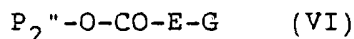
35

in which P' and m is as defined in claim 4 and TK'' is the radical



of trichokirin, as such, from which phenolic hydroxyl groups have been removed which consists in reacting, in an aqueous solution and at the ambient temperature a protein P_1 which is an antibody or an antibody fragment bearing at least one free thiol group attached to the said protein P_1 directly or via a spacing structure, with the 3-(2-pyridyl-disulfanyl)propionate of trichokirine.

13. Protein derivatives, when used for a process according to any one of claims 7 - 12, having the following statistical formula as hereinbefore defined:



in which:

- P_2'' represents the radical of a protein selected from the group comprising:

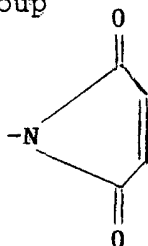
trichosanthin or trichokirin or any molecule derived from the said toxins by artificial modification of any one of their functional groups,

the said radical being the protein from which one or more phenolic hydroxyl groups of the tyrosines have been removed;

- the oxygen atom is that belonging to the phenolic hydroxyl groups missing from the radical P_2'' ; and

- E is as defined in claim 3, and

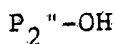
- G represent a group



or a -S-S-X group in which X is

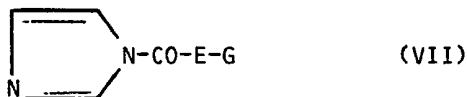
an activating group.

14. Process for the obtention of the protein derivatives as defined in claim 13 which consists in reacting a product of the formula:



in which P₂" is as defined above and the hydroxyl group is the phenolic hydroxyl missing from the tyrosines of the radical P₂", with a compound of the formula (VII) below :

05



at a temperature of 10 to 40°C, in an aqueous solvent optionally containing a water-miscible organic solvent, for example an ether solvent like dioxane or tetrahydrofuran.

10 15. Pharmaceutical compositions containing an immunotoxin as claimed in any one of claims 1 to 7 as the active principle.

Dated this 7th day of October 1987

SANOFI

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F.B. RICE & CO.