



AU9342746

(12) PATENT ABRIDGMENT (11) Document No. AU-B-42746/93
(19) AUSTRALIAN PATENT OFFICE (10) Acceptance No. 676679

(54) Title
PEPTIDE-CARBOHYDRATE CONJUGATES GENERATING T-CELL IMMUNITY

International Patent Classification(s)
(51)⁵ **C07K 007/06 A61K 037/00 A61K 037/02 C07K 007/08**
C07K 009/00
(51)⁶ **C07K 017/10**

(21) Application No. : **42746/93**

(22) Application Date : **23.04.93**

(87) PCT Publication Number : **WO93/21948**

(30) Priority Data

(31) Number	(32) Date	(33) Country
9201338	28.04.92	SE SWEDEN
9202553	07.09.92	SE SWEDEN
9203897	23.12.92	SE SWEDEN
9301141	06.04.93	SE SWEDEN

(43) Publication Date : **29.11.93**

(44) Publication Date of Accepted Application : **20.03.97**

(71) Applicant(s)
ASTRA AKTIEBOLAG

(72) Inventor(s)
MIKAEL JONDAL

(74) Attorney or Agent
E F WELLINGTON & CO , 312 St Kilda Road, MELBOURNE VIC 3004

(56) Prior Art Documents
WO 89/07448 40309/89

(57) Claim

1. A peptide/carbohydrate conjugate capable of generating T cell immunity against a carbohydrate structure, said conjugate comprising (i) a peptide component capable of binding a MHC class I molecule; and (ii) a carbohydrate component having the immunogenic specificity of said carbohydrate structure.

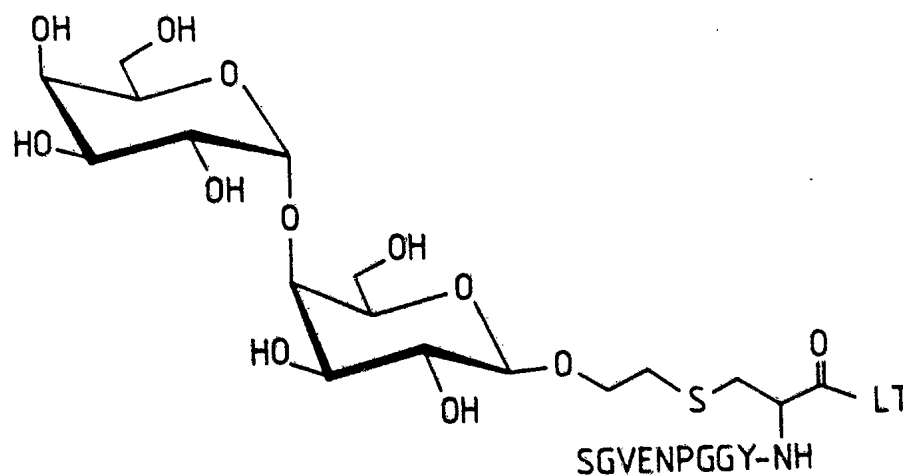


INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : A61K 39/00, 37/00, C07K 9/00		A1	(11) International Publication Number: WO 93/21948 (43) International Publication Date: 11 November 1993 (11.11.93)
(21) International Application Number: PCT/SE93/00353 (22) International Filing Date: 23 April 1993 (23.04.93) (30) Priority data: 9201338-2 28 April 1992 (28.04.92) SE 9202553-5 7 September 1992 (07.09.92) SE 9203897-5 23 December 1992 (23.12.92) SE 9301141-9 6 April 1993 (06.04.93) SE		(81) Designated States: AT, AU, BB, BG, BR, CA, CH, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, LK, LU, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published With international search report.	
(71) Applicant: <i>Astra Aktiebolag</i> AKTIEBOLAGET ASTRA (SE/SE); S-151 85 Södertälje (SE). (72) Inventor: JONDAL, Mikael ; Strandvägen 53, S-115 23 Stockholm (SE). (74) Agents: DANIELSSON, Sten et al.; Aktiebolaget Astra, Patent Department, S-151 85 Södertälje (SE).		 676679	

(54) Title: PEPTIDE-CARBOHYDRATE CONJUGATES GENERATING T-CELL IMMUNITY

Gal-Gal-SGV-12



(57) Abstract

The present invention relates to a novel class of biologically active compounds, to processes for their production and to their use in therapy. More particularly the invention provides immunogenic conjugates useful for generating T cell immunity against tumor-associated carbohydrate structures or against carbohydrate structures expressed on infectious agents and/or infected host cells. The said immunogenic conjugate comprises (i) a peptide component capable of binding an MHC class I molecule; and (ii) a carbohydrate component having the immunogenic specificity of said carbohydrate structure.

Peptide-carbohydrate conjugates generating
T-cell immunity

TECHNICAL FIELD

5

The present invention relates a novel class of biologically active compounds, to processes for their production and to their use in therapy. More particularly the invention provides immunogenic conjugates useful for generating T cell immunity against tumor-associated carbohydrate structures or against carbohydrate structures expressed on infectious agents and/or infected host cells.

BACKGROUND ART

15

Cell-mediated immunity

20

The vertebrate immune system is constantly active against invading microbes and malignant cells. It is well known that the adaptive immune system shows a much stronger response on second, as compared to first, encounter with an antigen. This fact is exploited in vaccination, which works by inducing a state of lasting immunity known as immunological memory. Immunological memory requires the activation of T lymphocytes, specific for the infectious agent. T lymphocytes detect infection within cells by recognizing — via the T-cell receptor (TCR) —

25

peptide fragments derived from the pathogen. However, most T lymphocytes are "MHC restricted", i.e. they recognize only complexes of peptides bound to highly polymorphic membrane proteins, encoded by class I and class II genes of the major histocompatibility complex (MHC) and presented on the surface on an accessory cell (designated an antigen-presenting cell or APC), in which the antigen has been

30

processed.

5 T lymphocytes can be classified as CD4⁺ or CD8⁺, depending on the specificity of an adherence receptor molecule. The CD4 adherence receptor recognizes MHC class II molecules, while CD8 binds class I. In addition, MHC restriction is further dependent on direct binding of the MHC molecule to certain parts of the TCR (Jorgensen *et al.*, 1992).

10 CD4⁺ T cells (Helper T cells) activate macrophages and antibody-producing B cells, while CD8⁺ T cells (Cytotoxic T cells, CTL) kill cells infected by viruses and intracellular bacteria. Antigens can be processed by one of two pathways, depending on their origin. In the first pathway foreign material from outside the cell is engulfed by a specialized antigen-presenting cell (often a macrophage or B-cell), which breaks down the material and links the processed antigen to class II MHC molecules. The complexes are transported to the cell surface and presented to Helper T cells. The second pathway is generally concerned with processing of proteins made within virus-infected or malignant cells. These proteins are processed in the cells, i.e. they are subjected to partial proteolysis so as to form peptide fragments. These fragments then associate with class I MHC molecules and are transported to the cell surface for presentation to Cytotoxic T cells.

25 The processing of antigens by separate pathways makes biological sense. Thus antigens taken up from the surroundings eventually elicit B cells to produce antibodies which will be capable of protecting the organism against a subsequent challenge by the exogenous antigen. On the other hand, in the case of antigens in the form of abnormal structures made within an abnormal or errant cell (for example a virus-infected or malignant cell), it is advantageous for the immune system to be activated with a result leading eventually to the killing of the errant cell.

30

MHC-binding peptides

In recent years there has been considerable progress in the analysis of peptides bound to MHC class I and II molecules (For reviews see *e.g.* Janeway, 1991; Rötzschke & Falk, 1991; Stauss, 1991; Tsomides & Eisen, 1991). Thus it has been found that MHC class I molecules bind short peptides of only about 8-12 amino acids and that MHC class II molecules bind peptides of about 10-17 amino acids.

Furthermore it has been found that the peptide fragments resulting from processing of antigens are transported to the cell surface bound in a groove on the extracellular part of an MHC molecule. For MHC class I molecules it has been found that individual amino acid side-chains, located at precise positions along the peptide, bind into the peptide-binding groove (Madden *et al.*, 1991). The position of these pockets and the amino acids that line them can be different for different allelic variants. Consequently, different MHC class I molecule can bind a different set of peptides, and allele-specific motifs have been found for various MHC alleles (Van Bleek & Nathenson, 1990; Falk *et al.*, 1991; Jardetzky *et al.*, 1991). For example, peptides binding HLA-A2.1 preferably have L or M in position 2 and V or L in position 9, *i.e.* in the C-terminal position (Rötzschke & Falk, 1991).

Examples of peptides capable of binding MHC class I molecules in the murine system are A S N E N M E T M and S G P S N T P P E I, (SEQ ID NOS: 1 and 2), which both are presented by H-2-D^b molecules. An example of a peptide capable of binding an MHC class I molecule in the human system is G I L G F V F T L, (SEQ ID NO: 3), which is presented by HLA-A2.1 molecules (Falk *et al.*, 1991).

Conjugation of a MHC (class II) binding peptide, D Y G I L Q I N S R, (SEQ ID NO: 19), to a carbohydrate, 4-O- α -D-galactopyranosyl- β -D-galactopyranose, is described in Elofsson *et al.* (1991).

WO 89/07448 discloses compositions for modulating the immune response of a host, employing peptides having homology with peptides binding MHC class I molecules.

5 Synthetic peptides as carriers for T cell specific epitopes

CTL:s can be generated from spleens of mice painted with trinitrochlorobenzene (TNP), and selected for killing of TNP-coated syngenic target cells. Some CTL:s generated like this have been shown to recognize short MHC class I (K^b)-bound peptides of 10 amino acids with TNP bound to an internal (position 6) lysine residue. Furthermore, 10 some CTL:s kill syngenic target cells that have processed and presented TNP bound to different carrier proteins (MSA, BSA and KLH) and also TNP-coated, allogenic target cells (Ortmann *et al.*, 1992)

15 Tumor-associated antigens

The possibility that tumors may be recognized as foreign by the immune system (based on the abnormal character of tumor cells) would offer valuable opportunity for developing effective cancer therapies. In experimental systems tumors have shown to be highly immunogenic and 20 the triggered immune responses was sufficient to eliminate the tumor cells. In the clinical situation, however, a tumor, being a product of multiple genetic and adaptive alterations, has little immunogenicity at the time when it becomes a medical problem. Accordingly, few truly tumor associated protein antigens, *i.e.* antigens being expressed in tumor cells 25 only, have been found.

In contrast to the lack of tumor associated protein antigens, a variety of abberant carbohydrate (CHO) structures are present on tumor cells (for a review see Hakomori, 1991). These are formed as a consequence of 30 abnormalities in the enzyme systems responsible for the assembly of the CHO chains. The chains are often truncated with the result that CHO

epitopes (which are absent or hidden on normal cells) are expressed on tumor cells.

5 Aberrant CHO structures on tumor cells can exist in the form of glycoprotein, glycolipid or a complex of both of these two forms. Glycoproteins are often secreted into body fluids whereas glycolipids to a large extent are membrane bound.

10 Examples of tumor associated carbohydrate antigens are the GM3 ganglioside, which has been identified in mouse melanoma B16 cells (Nores *et al.*, 1987), GD3 ganglioside associated with human melanoma cells (Portoukalian *et al.*, 1979) and Gb3 ganglioside which is expressed in Burkitt lymphoma cells (Wiels *et al.*, 1981; Brodin *et al.*, 1988).

15 Carbohydrates associated with infectious diseases

Carbohydrates associated with infectious diseases can be expressed on the infectious agents, on secreted or shed material from these, or on the surface of infected host cells.

20 There are some examples of verified, or putative, carbohydrate-specific T cell responses, associated with diseases caused by infectious agents like *Salmonella typhimurium* (Robertsson *et al.*, 1982), *Leishmania major* (Moll *et al.*, 1989), *Candida albicans* (Domer *et al.*, 1989) and *Mycobacteria* (Crowle, 1988).

25 Carbohydrate antigens associated with HIV infection are described in Hansen *et al.* (1990).

Immunotherapy against tumors

30 Immunotherapy against tumors has a long medical history and has been tried by both non-specific and specific means. In non-specific manipulations, the immune system has been activated by various factors

such as BCG, IL-2 *etc.*, in order to increase the existing background immunity against the tumor. In specific manipulations, vaccines have been constructed using tumor cells or material derived from these.

5 T cell-mediated immune responses play crucial roles in antitumor immunity. Whereas the mechanisms of activation of T cell responses against proteins are characterized to some extent (see above), they are essentially unknown for carbohydrates. Carbohydrate antigens are poor candidates for presentation to MHC molecules, because of structural
10 constraints imposed by the peptide-binding groove of the MHC proteins. Nevertheless, Ishioka *et al.* (1992) has shown that the CHO moiety can be an important part of the antigenic determinant recognized by T cells, and suggest that although there are limitations to the generation of CHO-specific T cell responses, such responses could be generated by
15 judicious placement of the carbohydrate within a MHC-binding peptide.

Longenecker and coworkers (WO 88/00053; Henningson *et al.*, 1987) describe the use of "Synthetic Tumor-Associated Glycoconjugates" (S-TAGs) for stimulation of anticancer T cell immunity. In these experiments,
20 synthetic Thomsen-Friedenreich (TF) and Tn antigens, carbohydrates expressed on most human adenocarcinomas, were conjugated to a carrier protein and used to demonstrate that delayed-type hypersensitivity (DTH) effector cells could recognize and respond to the carbohydrate determinants. An S-TAG composed of TF antigen coupled to carrier
25 (keyhole limpet hemocyanin) and emulsified in Ribi adjuvant was administered to mice with TA3-Ha adenocarcinoma. When administration was preceded by treatment with cyclophosphamide, a 50-90% long term survival of the hosts was observed (Fung *et al.*, 1990).

30 Thurin *et al.* (1991) describe a conjugate comprising (i) a tumor-associated carbohydrate hapten; (ii) a peptide containing a viral mouse Helper T cell epitope; and (iii) a Quil-A glycoside based adjuvant. Mice

immunized with the conjugate showed IgM responses specific for the hapten together with a Helper T cell reactivity to the viral epitope.

5 PURPOSE OF THE INVENTION

A main object of the invention is to provide a means for generating T-cell immunity against a carbohydrate structure, in particular antitumor immunity against carbohydrate antigens.

10

Although the prior art suggests that antitumor immunity against carbohydrate antigens may be raised with a synthetic vaccine, in practice the achievement of this aim has remained elusive. For example Longenecker's S-TAGs (described in the section above) utilize a

15 conventional protein as a carrier of the carbohydrate structure. Such a glycoprotein has to be proteolytically cleaved before it can be presented on the cell surface, which means that there is no control over what is finally presented to TCR. In addition, a large carrier protein contains several different epitopes, competing for T cells.

20

Further, despite major research, prior art efforts to develop immunological therapies for cancer have had disappointing results. Also, prior art procedures for stimulating immune responses against carbohydrate-associated antigens have had limited success, especially in cases where

25 it is desired to stimulate an immune response against a disease-associated carbohydrate structure, for example one expressed on tumor cells, but not on normal cells.

30

The present invention rises above the prior art by providing a novel class of conjugates comprising MHC class I binding peptides together with a carbohydrate structure which can be used, *inter alia*, in the development of synthetic vaccines for eliciting anti-tumor immunity. Further the

conjugates according to the invention are capable of generating cytotoxic T cell, instead of helper T cell, responses against such carbohydrate structures. Further the conjugates according to the invention permit a higher degree of control (1) over the identity of presented epitope and (2) over which T cell clones will be expanded.

A further object of the present invention is to enable the production of carbohydrate-specific T cells, *i.e.* cytotoxic T cells generated by the use of class I MHC-binding peptides. The carbohydrate-specific T-cells generated according to the invention need not be MHC-restricted. Accordingly, cells generated in one individual can react with cells from another individual, from the same or even from another species.

The generation of carbohydrate-specific T-cells according to the invention is thus quite different from prior art experiments where a generated Helper T cell response is directed to a peptide epitope, as where haptens are conjugated to carrier proteins in order to achieve antibody response against the hapten or where Thurin *et al.* extended this concept to the use of defined MHC class II-binding peptides.

DISCLOSURE OF INVENTION

As indicated, the purpose of the invention has been achieved by conjugating a suitable peptide capable of binding a MHC class I molecule, to a suitable carbohydrate structure. By conjugating the carbohydrate to the peptide in an optimal position, the carbohydrate antigen can have a high affinity to TCR, which can make the response independent of adherence receptors, *i.e.* the T cell will not be MHC restricted. This will create CHO-specific responses from both cytotoxic, as well as helper, T cells.

Fields of use

The conjugates according to the invention can be used for treatment of malignant diseases, including melanoma, breast cancer, lung cancer and gastrointestinal cancer. They can further be used for treating diseases by infectious agents causing expression of the carbohydrate target molecule.

The conjugates of the invention can be used for inducing T cell responses *in vivo* in order to eliminate tumors and/or infections. They can also be used for inducing T cell responses *in vitro*, using an extra-corporal stimulation procedure for later reinfusion of activated T cells into patients.

Depending on the type of immune response achieved, the conjugates can be administered once or if necessary by continuous, regular immunizations.

STATEMENTS OF INVENTION

According to a first aspect of the invention there are provided conjugate capable of generating T cell immunity against a carbohydrate structure, said conjugate comprising (i) a peptide component capable of binding a MHC class I molecule; and (ii) a carbohydrate component having the immunogenic specificity of said carbohydrate structure.

The invention further provides a method of stimulating the production of cytotoxic T cells (CTLs) in a patient, wherein said CTLs have the potential to destroy or attenuate cells presenting a characteristic disease-associated carbohydrate structure, which comprises administering to the patient an effective dose of a conjugate as herein defined.

Also provided according to the invention is a method of producing cytotoxic T cells (CTLs) which have the potential to destroy or attenuate disease-associated cells presenting a characteristic disease-associated

carbohydrate structure, which comprises contacting a cell population with a conjugate as herein defined, wherein said cell population includes (a) cells possessing MHC class I molecules capable of binding to the peptide component of said conjugate and (b) cells capable of being converted to CTLs having the said potential on interaction with cells (a) having said conjugate bound to a MHC class I molecule.

CTLs may be produced in the manner described by administering the peptide/carbohydrate conjugate to an animal whereby the CTLs are produced *in vivo*. Alternatively the CTLs may be produced *in vitro* by contacting cells which have been removed from the animal body with the peptide/carbohydrate conjugate. The so-produced CTLs may then be administered (e.g. by introducing them into the same or a different animal) as part of a therapeutic regime.

The invention further provides the use of the peptide/carbohydrate conjugates described herein for the manufacture of pharmaceutical compositions.

More specifically there is provided the use of the conjugates for the manufacture of a pharmaceutical composition for inducing a desired immunological state in a patient, wherein said immunological state results from an interaction between said conjugate and an MHC class I molecule whereby a cellular component of the immune system is stimulated to induce a response specifically associated with said carbohydrate structure.

Preferably the peptide component and the carbohydrate component and the carbohydrates according to the invention have the characteristics set forth in the following paragraphs.

The peptide component

An important feature of the peptide/carbohydrate conjugates of the invention is that the peptide component thereof should be capable of binding a MHC class I molecule. For a given peptide, the capability to bind a MHC class I molecule may be determined in a number of ways. Thus for example the peptide may have certain selected structural characteristics such as size or the presence of particular amino acids residues in specified positions. Alternatively or additionally the capability of a candidate polypeptide to bind a MHC class I molecule may be assessed empirically by carrying out one or more immunological assays. As a further alternative, the peptide component of the peptide/carbohydrate conjugates of the invention may have a sequence or motif which is substantially identical to the sequence of a peptide which has been isolated by dissociating a naturally-occurring complex between a peptide and a MHC class I molecule. Each of these indicators of the capability of a peptide to bind an MHC class I molecule will be described in further detail below.

Particular reference is made to three articles (Fremont *et al*, 1992 Matsamura *et al* 1992 and Latron *et al*) appearing in Science, Vol. 257, 14th August 1992.

These articles describe the characteristics of peptide motifs which are capable of binding MHC class I molecules with particular reference to the murine MHC class I molecule H-2K^b and the human class I molecule HLA-A2 (the latter being present in approximately 40% of the human population).

A characteristic of MHC class I molecules which is associated with their capacity to bind a range of characteristically recognised peptides is the presence of a peptide binding groove which defines a series of generally six so-called "pockets" which accommodate or anchor structural elements

of the peptides which are capable of being bound. The peptide binding groove or cleft is limited laterally by two α -helixes while the floor is defined by a β -pleated sheet.

5 Of the six pockets (normally designated A, B, C, D, E and F) generally two are arranged to accommodate the $-NH_2$ and $-COOH$ termini of the peptide respectively.

10 The peptide component of the conjugate is preferably chosen from peptides having the optimal number of amino acids for binding MHC class I molecules, *i.e.* 5-25 amino acids, especially 8-12 amino acids, the size being selected to allow for proper binding in the peptide binding groove. The optimal size of the peptide to enable efficient binding in the groove of the MHC class I molecule is 8 or 9 amino acids, more preferably, with 9 amino acids. Still more preferably the peptide component has
15 the amino acid V, I, L or T in position 6, most preferably the amino acid V in position 6, and/or the amino acid V or L in the C-terminal position, most preferably the amino acid L in the C-terminal.

20 Thus, the peptide component of the conjugates of the invention preferably have a hydrophobic amino acid in the C-terminal position to facilitate binding in pocket F. Other amino acids of the peptide component are preferably selected so as to fit within other pockets in the peptide binding cleft. Selection of suitable and preferred amino acid residues may be aided by computer-based molecular modelling techniques as described e.g. by Masazumi (1992).

25 The peptide can be synthesized by known methods. The immunogenicity of the peptide part of the conjugate can be further increased by chemical modifications. Conjugates comprising such modified peptides are part of the invention.

Peptides to be synthesized can be chosen from those known from the literature or from those which immunogenicity are determined by known methods. In the latter case peptides can be isolated from whole-cell



lysates or from purified MHC molecules and separated by *e.g.* high-performance liquid chromatography. Optimal binding of the peptide to the MHC molecule is reflected as high stability of the peptide-MHC complex on the surface of antigen-presenting cells.

5

The invention further provides a process for producing a peptide/carbohydrate conjugate as defined above which comprises one or more of the following procedure steps:

10

- (a) synthesizing the peptide component of the conjugate by a known technique of peptide synthesis,
- (b) synthesizing the carbohydrate component of the conjugate by a known technique of carbohydrate synthesis,
- (c) protecting one or more -COOH, OH, -NH₂ or -SH groups of the peptide component prior to coupling the peptide component covalently to the carbohydrate component,
- (d) protecting one or more -OH, -COOH, -NH₂, -CHO or =CO groups of the carbohydrate component prior to coupling the carbohydrate component covalently to the peptide component,
- (e) activating an unprotected -OH, -COOH, -NH₂, -CHO, or =CO group of the carbohydrate component,
- (f) activating an unprotected -OH, -COOH, -NH₂, or -SH group of the peptide component,
- (g) reacting at least one of the carbohydrate component and the peptide component with a bifunctional linking reagent,
- (h) reacting the carbohydrate component and the peptide component covalently so as to form the desired conjugate, said components being suitably protected, activated and/or reacted with a bifunctional linking reagent,
- (i) subjecting an intermediate protected peptide/carbohydrate conjugate to a deprotecting procedure.

15

20

25

30

In the present context, a peptide "capable of binding a MHC class I molecule", may consequently be further defined as a peptide whose complex with a MHC class I molecule will be stable on the surface of antigen-presenting cells.

5

Stability of the above mentioned complex can be measured by *in vitro* assays known to a person skilled in the art. It can be measured as long-term expression on the cell surface, due to a slow decomposition of the complex. Another characteristic reflecting high stability is the sensitization of target cells for corresponding CTL:s at very low concentration of the peptide. Stability can also be reflected as the capacity of peptides to upregulate the expression of the corresponding class I molecule on certain target cells *in vitro* at low molar concentrations.

10

15 Stability is also related to high immunogenicity *in vivo*, when short peptides are directly injected into mice.

The capacity of a glycopeptide to be bound by an MHC class I molecule may be assessed by carrying out immunological assays designed to detect one or more of the following characteristics

20

(1) glycopeptide binding to soluble MHC-I leading to the formation of complexes which may, for example, be detected by configuration-dependent monoclonal antibodies, or by association with labelled β_2 -micro-globulin, using conventional biochemical techniques;

25

(2) expression of the CHO part after the glycopeptides have bound to empty MHC-I molecules on the surface of mutant host cells (such as RMA-S and T2 cells). The CHO part may then be detected by CHO-specific monoclonal antibodies such as MC2102 (Gal₂ specific).

30

(3) upregulation of the corresponding MHC-I restriction element by glycopeptides, using MHC-I specific monoclonal antibodies.

(4) the recycling capacity of the glycopeptides (see Section 3 below
- "EXTERNAL MHC-1 BINDING PEPTIDES ARE RECYCLED TO
THE CELL SURFACE AFTER INTERNALIZATION")

5 In order to carry out these assays use may be made of available
antibodies which are capable of detecting MHC class I molecules as well
as antibodies which are capable of detecting specific carbohydrates. A
particular example of the latter is the commercially available monoclonal
antibody MC2102 (New Biocarb, Lund, Sweden) which is capable of
10 detecting the carbohydrate Gal₂.

These procedures may be used to assess the capacity of a given peptide
to be bound by MHC class I molecules in the following manner.

15 First a glycopeptide is synthesised in which Gal₂ is covalently linked to
the peptide. The glycopeptide may then be tested for its capacity to bind
to MHC class I molecules on target antigen-presenting cells, and for the
carbohydrate part to be expressed, i.e. bound in a configuration in which
the Gal₂ epitope is located in a position in which it can be detected by a
20 specific antibody. Additionally upregulation of the corresponding MHC-I
restriction element may be assessed by determining the capacity of the
antigen-presenting cells to interact with anti-MHC class I antibodies.

The capacity of the conjugate to be recycled may be assessed by
25 incubating the cells (with bound conjugate) at 37°C whereupon the
glycopeptide will internalise. The temperature may then be lowered and
the cells treated with a proteolytic enzyme (e.g. Pronase) to strip off
exposed peptide/carbohydrate conjugate. On reheating, recycled
conjugate may be detected by probing for the Gal₂ epitope using the
30 anti-Gal₂ antibody.

In a further test, the conjugate can be tested in immunisation experiments and assessed for its capacity to stimulate the formation of CTLs. In control experiments the generated CTLs can be tested against the peptide component (without the carbohydrate component) to demonstrate that the observed CTL-stimulation is associated with the carbohydrate (see "Experimental Test System" below).

The carbohydrate component

The carbohydrate part of the conjugate may be synthesized by known methods and is preferably chosen from carbohydrate structures that:

- (a) are stably expressed on the target cell so that the same part always is exposed outwards to the T cell receptor;
- (b) have a high density on the target cell so that the T cell receptor can be triggered independently of MHC restriction and adherence receptors; and
- (c) have a high membrane association so that secretion into the general circulation is avoided. Soluble carbohydrates are, in contrast, likely to create problems because of induction of peripheral suppressor T cells and blocking of the generation of protective immunity.

A further advantage with choosing a carbohydrate with high expression on target cells is that such high expression may lead to secondary modifications in the membrane which increase the target cell specificity of the exposed CHO epitopes.

The carbohydrate should be sized to enable the T cell receptor to encompass a CHO epitope. A preferable size, to which the invention however is not restricted, is half the length of the peptide.

Although single sugar moieties are not excluded, preferably the carbohydrate component of the conjugates of the invention will contain at

least two linked sugar moieties, i.e. it will be a di-saccharide or oligo saccharide. The precise number of linked sugar moieties will be dependent on the immunogenic specificity of the carbohydrate structure, but 3, 4, 5 or more linked sugar moieties may be present.

5

Preferably the upper limit on the size of the carbohydrate component will be dictated by the requirement that it should be sized to enable a T cell receptor to simultaneously encompass an epitope of the carbohydrate structure and also the Class 1 restriction element. Typical upper limits are not more than 10 sugar moieties, preferably not more than 8 and most preferably not more than 5. Preferred ranges are thus from 1 to 10, more preferably from 1 to 8 and most preferably from 1 to 5 sugar moieties. For each of these preferred ranges, the lower limit of 1 may be increased to 2, 3, 4 or 5 if desired.

10

15

The individual sugar moieties are preferably selected from aldopentoses, ketopentoses, aldohexoses and ketohexoses. These may be in linear or cyclised form and if desired, derivatised by oxidation, reduction, amination, acetylation or any combination of these. Oxidation can include conversion of one or more $-CH_2OH$ groups to $-CHO$ or $COOH$ groups, as well as conversion of a $-CHOH-$ group to a $-CH_2-$ group. Reduction can include any of the reverse conversions. The aldopentoses include D-ribose, D-arabinose, D-xylose and D-lyxose as well as other stereo-isomers thereof, i.e. the L-isomers. The aldohexoses include D-allose, D-altrose, D-glucose, D-mannose, D-gulose, D-idose, D-galactose and D-talose as well as other stereo-isomers thereof, i.e. the L-isomers. The ketopentoses include D-ribulose and D-xylulose as well as other stereo-isomers thereof, i.e. the L-isomers. The ketohexoses include D-psicose, D-fructose, D-sorbose and D-tagatose as well as other stereo-isomers thereof, i.e. the L-isomers. When cyclised, the sugar moieties can be in the α - or β - configuration and (if appropriate) in the pyranose or furanose forms.

20

25

30

Most preferably the carbohydrate component contains one or more sugar moieties selected from ones normally found in the carbohydrate portions of glycolipids and glycoproteins. These include β -L-fucose (Fuc), β -D-galactose (Gal), β -D-N-acetylgalactosamine (Gal NAC), β -D-N-acetylglucosamine (Glc NAC), β -D-mannose (Man), neuramic acid (Neu) and sialic acid (Sia).

Carbohydrate components corresponding to the CHO portions of the globo/ganglio series of glycolipids are further examples of suitable tumor targets, as they are mostly membrane-associated and are not secreted. (see Oettgen, 1989; Chapter 6 "General Concept of Tumor-associated Antigens: Their Chemical, Physical, and Enzymatic Basic").

The mode of linking adjacent sugar moieties is not critical, i.e. linkages can be effected utilising any of carbon atoms 1 to 5 of pentoses and any of carbon atoms 1 to 6 of hexoses. Further the linkages can be either α or β . Most preferably the linkages involve carbon atoms 3 and 4 with the β configuration being preferred.

The carbohydrate component and the peptide component can be conjugated (or linked covalently together) by any convenient means. Where the peptide component includes an amino acid bearing a hydroxyl group (i.e. as in serine, threonine or tyrosine) the linkage can be via an O-glycoside bond. Where the peptide component includes an amino acid containing an -NH_2 side chain (as in asparagine) the linkage can be via an N-glycoside linkage.

Alternatively the linkage can involve the introduction of a so-called "spacer arm" between the carbohydrate component and the peptide component. Thus, for example where the peptide component includes an amino acid (cysteine) having an -SH side chain, the carbohydrate

component can be linked to the -SH side chain via a $-(C_nH_{2n})-O-$ linkage wherein n is >2 , preferably 2 to 4.

- 5 The location of the carbohydrate component on the peptide/carbohydrate conjugate is preferably chosen so that when bound to an MHC class I molecule, the carbohydrate component occupies a generally central location in the peptide binding groove, with at least part of the carbohydrate component protruding from the groove in a manner enabling it to be presented to a CTL.

10

Examples of Carbohydrates suitable for conjugation

- 15 Examples of carbohydrate structures, associated with human tumors or infectious diseases, and suitable for conjugation are given below in Table 1. The indicated tumors exemplify types of cancer susceptible to treatment with the conjugates of the present invention. For the purpose of this description, the term "carbohydrate structures" is to be understood as comprising derivatives of the exemplified carbohydrates, such as e.g. lactones and lactams.

TABLE 1

1. CARBOHYDRATES ASSOCIATED WITH HUMAN TUMORS:

5

1.1 The majority of tumors:Gal β 4Glc β Cer

Lactosylceramid

1.2 Melanoma:

10

NeuAc α 8NeuAc α 3Gal β 4Glc β Cer

GD3

9-O-Ac-GD3

NeuAc α 8NeuAc α 3(GalNAc β 4)Gal β 4Glc β Cer

GD2

9-O-Ac-GD2

Lactonized forms of these

15

1.3 Colon cancer and other types of cancer:GalNAc α -Ser(Thr) (glycoprotein)

Tn-antigen

NeuAc α 6GalNAc α -Ser(Thr)

Sialyl-Tn-antigen

Gal β 3GlcNAc

Type 1 chain

20

NeuAc α 3Gal β 3(Fuc α 4)GlcNAc β

Sialyl-Lewis a

NeuAc α 3Gal β 3(Fuc α 4)[NeuAc α 6]GlcNAc β

Disialyl-Lewis a

Gal β 3(Fuc α 4)GlcNAc β Gal β 3(Fuc α 4)GlcNAc β

Dimer Lewis a

NeuAc α 3Gal β 4(Fuc α 3)GlcNAc β

Sialyl-Lewis x

Gal β 4(Fuc α 3)GlcNAc β 3Gal β 4(Fuc α 3)GlcNAc β

Dimer Lewis x

25

Gal β 4(Fuc α 3)GlcNAc β 3Gal β 4(Fuc α 3)GlcNAc β 3Gal β 4(Fuc α 3)GlcNAc β

Trimer Lewis x

NeuAc α 3-Dimer Lewis xNeuAc α 3-Trimer Lewis xNeuAc α 6-Oligomer Lewis x

30

Lactonized forms of these in the case of sialic acid

- 1.4 Lung cancer and other types of cancer:
 Gal β 4GlcNAc β 3Gal β 4GlcNAc β i-antigen (rep. lactosamine)
 Gal β 3(Fuc α 4)GlcNAc β Gal β 4(Fuc α 3)GlcNAc β Lewis a - Lewis x
 5 Fuc α 2Gal β 3GalNAc β 4(NeuAc α 3)Gal β 4Glc β Cer Fuc-GM1
 Lactonized forms of Fuc-GM1
- 1.5 Burkitt's Lymphoma:
 Gal α 4Gal β 4Glc β Cer Gb3
 10
- 1.6 Breast Cancer:
 Fuc α 2Gal β 3GalNAc β 3Gal α 4Gal β 4Glc β Cer Fuc-Globopenta
 Tn-antigen
 Sialyl-Tn-antigen
 15
- 1.7 Teratocarcinoma:
 NeuAc α 3Gal β 3GalNAc β 3Gal α 4Gal β 4Glc β Cer Sialyl-Globopenta
 Lactonized form of the above
- 20 2. CARBOHYDRATES ASSOCIATED WITH EXPERIMENTAL TORS
- 2.1 Melanoma (Hamster):
 NeuAc α 3Gal β 4Glc β Cer GM3
 25 GD3
 9-O-Ac-GD3
 GD2
 Lactonized forms of these
- 30 2.2 Melanoma (Mouse):
 GM3
 Lactonized GM3

3. CARBOHYDRATES ASSOCIATED WITH INFECTIOUS AGENTS

Mannan from *Candida albicans*

Polysaccharide isolates from *Mycobacterium bovis* strain BCG

5 Lipophosphoglycan from *Leishmania major*

O-antigenic polysaccharides from *Salmonella typhimurium*

4. CARBOHYDRATES ASSOCIATED WITH HIV

10	Fuca α 2gal β 4(Fuca α 3)GlcNAc β	Lewis y
	GalNAc α 3(Fuca α 2)Gal β 3GlcNAc β	Blood group A ₁
	NeuAc α 6GalNAc α -Ser(Thr)	Sialyl T _n antigen
	GalNAc α -Ser(Thr)	T _n antigen

15

Preferred characteristics of the conjugate

The carbohydrate can be coupled to the carrier peptide aminoterminally, carboxyterminally, or it may be coupled to internal amino acids. As indicated, internal coupling is preferred.

20

Also, as described above, specific anchoring amino acids, binding in pockets on the MHC molecule, have been defined in the literature for peptides binding various MHC molecules. These anchoring amino acids should preferably not be used for conjugation to the carbohydrate structure.

25

The synthetic carbohydrate should have a high degree of molecular stability when conjugated to the carrier peptide, in order to expose the carbohydrate epitopes with high specificity. The position of the carbohydrate in the conjugate should be such that the T cell receptor can recognize a CHO epitope, in order to generate optimal triggering of T cells.

30

Further, the size and position of CHO should be optimal for recognition by TCR, especially by the third complementarity determining region (CDR3). A preferable position, to which the invention however is not restricted, is centrally in the glycopeptide.

5

The CHO and peptide may be conjugated via optional linker elements. Such linker elements can be additional amino acids, *e.g.* cysteine, or other suitable chemical compounds.

10 Experimental test system

The immunogenicity of the conjugates of the invention can be tested in immunization experiments known to a person skilled in the art. CHO specificity can be analyzed by "criss-cross" experiments: T-cells that respond to the conjugate "CHO-A", wherein A designates a peptide, are tested with CHO-A; peptide A alone; CHO-B, wherein B designates a peptide different from A; and peptide B alone. If *e.g.* CTL responding to CHO-A also kill cells presenting CHO-B, but not cells presenting peptides alone, the response can be concluded to be CHO-specific. In addition, T cells may be tested against target cells expressing the CHO part in the form of a glycolipid.

15
20

Pharmaceutical formulations

The conjugates according to the invention can be administered in conventional dosage forms. When administered in the form of vaccines, it is preferred that they include one or more suitable adjuvants.

25

It has been found that administration of peptide/carbohydrate conjugates according to the invention can result in binding of the conjugates to empty MHC molecules on the cell surface. Such a binding is possible since the glycopeptide has in effect been synthetically pre-processed, *i.e.* it does not have to enter the cytosol to be processed.

30

Alternatively, the conjugates can be incorporated into a structure able to facilitate transport into the cytosol, in order to achieve early binding to the MHC molecule. Such structures can be liposomes or "immunostimulating complexes" (iscoms) (Morein *et al.*, 1984).

5

The following examples illustrate the synthesis of conjugates according to the invention.

EXAMPLES

10

1. PREPARATION OF GLYCOCONJUGATES

1.1. Synthesis of glycopeptides

15

Glycopeptides can be synthesized by *e.g.* coupling preformed spacer arm glycosides with peptides containing a thiol function.

20

Thus, a cysteine moiety was introduced in the peptide sequence and the nucleophilic properties of the cysteine sulphur atom was utilized to create a thioether linkage between the peptide and an electrophilic spacer arm glycosidically linked to the carbohydrate moiety. The spacer arms used were the 3-bromo-2-bromomethylprop-1-yl (DIB) (Magnusson *et al.*, 1982) and 2-bromoethyl (Dahmén *et al.*, 1983 b) groups. By this strategy it was possible to couple carbohydrates representing various epitopes and antigenic determinants to the same peptide sequence. The latter could in turn be obtained by standard peptide synthesis techniques and assayed for biological activity prior to conjugation. Thus, time required for peptide syntheses was kept at a minimum.

25

The coupling reactions were performed in dimethylsulfoxide or N,N-dimethylformamide.

30

Prior to coupling, hydrogen bromide was eliminated from the DIB group using N,N,N,N-tetrabutylammonium fluoride (Magnusson *et al.*, 1982). The allylic bromide obtained was then reacted with the cysteine containing peptide. Due to side reactions, such as formation of unreactive allylic fluorides, this method did not allow the excess of the carbohydrate derivative used to be recovered.

Cesium carbonate was used as a promoter when 2-bromoethyl glycosides were employed (Dahmén *et al.*, 1983 b).

The amount of N,N,N,N-tetrabutylammonium fluoride or Cesium carbonate needed for coupling was dependent on included trifluoroacetic acid in the peptides used. The amount of trifluoroacetic acid included varied depending on the batch and structure of the peptide. The choice and amount of solvent was determined by the solubility properties of the peptide.

The coupling reactions were monitored by HPLC and were quenched by the addition of water containing 0.1% trifluoroacetic acid. The resulting glycopeptides were purified by preparative HPLC and the molecular weights of the products were confirmed by FAB MS.

Both saturated and allylic bromides have been reported to react with cysteine thiols in unprotected peptides to give, as major products, S-alkylated peptides.

Yang *et al.* (1991) prepared farnesylated peptides by reacting cysteine containing octa- and pentadecapeptides with farnesyl bromide at pH 6-9 in mixtures of N,N-dimethylformamid/dimethylsulfoxid/acetonitril. N,N-diisopropylethylamine was used as a promoter. Exclusive S-alkylation was reported even at pH 10-12.

Selective S-alkylations of cysteine-containing peptides was also performed (Or *et al.*, 1991) in methanolic or ethanolic ammonia using various alkylating agents, *e.g.* a primary bromopropyl derivative.

5 1.2. Synthesis of the spacer arm glycosides (Figures 1 and 2)

The spacer arm glycosides used for coupling are exemplified by the DIB β -glycoside and the 2-bromoethyl β -glycoside of NeuNAc α 3Gal β 4Glc (3''-sialyllactose), the 2-bromoethyl β -glycosides of Gal α 4Gal and Gal α 4Gal β 4Glc and the 2-bromoethyl glycoside of "GM3 Lactam" (Compounds 9, 15, 28 48 and 55 respectively).

DIB β -glycoside of 3''-sialyllactose (9) was synthesized in eleven steps from 2-trimethylsilylethyl 2,3,6-tri-O-acetyl-4-O-(2,3,4,6-tetra-O-acetyl- β -D-galactopyranosyl)- β -D-glucopyranoside (1) (Jansson *et al.*, 1988).
15 Deacetylation of (1) gave (2) which was isopropylidenated and benzylated to give the benzyl protected 3',4'-O-isopropylidene lactose derivative (3). Hydrolysis of the 3',4'-acetal gave the glycosyl acceptor (4) in an overall yield of 51%. Glycosylation (Marra and Sinaÿ, 1990) of the
20 lactose derivative (4) in acetonitrile at -23°C using the acetyl protected α -xanthate (5) (Marra and Sinaÿ, 1989) of sialic acid methyl ester as a glycosyl donor and dimethyl(methylthio)sulfonium trifluoromethanesulfonate (DMTST) as a promoter gave, after
25 debenzylation and acetylation, the trisaccharide derivative (6) in a yield of 17.5%. The ease of purification and the yield of (6) were slightly improved when acetylation of unreacted hydroxy functions of the reaction products was performed before hydrogenolysis of the benzyl ethers. The
30 2-trimethylsilylethyl glycoside (6) was converted (Jansson *et al.*, 1988) to the corresponding 1 β -acetate (7) which was used as a glycosyl donor in a Lewis acid catalyzed glycosylation of 3-bromo-2-bromomethylpropan-1-ol yielding the protected β -glycoside (8) in 46% yield. Deprotection of (8) by methanolic sodium methoxide followed by aqueous methanolic sodium

hydroxide gave the DIB β -glycoside of 3''-sialyllactose (9) which, after elimination of HBr to give the corresponding 2-bromomethylprop-2-en-1-yl glycoside, was used for coupling to peptides.

- 5 The 2-bromoethyl β -glycoside of 3''-sialyllactose (15) was in turn synthesized from the acetyl protected 2-bromoethyl glycoside of lactose (10) (Magnusson *et al.*, 1982) by a five step sequence. Deacetylation of (10) gave (11) which was isopropylidenated (Catelani, 1988) to give the 3,4'-O-isopropylidene derivative (12) in 86% yield. Partial benzylation
10 (Murase *et al.*, 1989) at -60°C followed by hydrolysis of the 3,4'-acetal gave the 2,6,6'-tri-O-benzoate (13) in 31% yield. The lactose derivative (13) was glycosylated -60°C (Lönn and Stenvall, 1992) using the xanthate (5) as a glycosyl donor and methylsulfonium trifluoromethanesulfonate (MST) as a promoter to give the protected
15 trisaccharide derivative (14) in a yield of 75%. Deprotection as for 8 gave the 2-bromoethyl glycoside (15) in 96% yield.

1.3. Experimental

- 20 ^1H - and ^{13}C -NMR spectra were recorded in CDCl_3 at 300 and 75 MHz respectively on a Varian Gemini 300 spectrometer unless otherwise stated. Signals from undeuterated solvent at 7.25 and 77.0 ppm respectively were used as internal reference signals.

- 25 Optical rotations were measured using a Perkin Elmer 241 polarimeter.

- Thin layer chromatography was performed on Merck DC-Fertigplatten (Silica gel 60 F₂₅₄ 0.25 mm) and spots visualized by spraying with 10% sulphuric acid followed by charring at elevated temperature and/or
30 spraying with molybdotophosphoric acid/ CeSO_4 /dil. H_2SO_4 followed by heating (Renaud and Seebach, 1986). MatrexTM Silica Si 0.35-0.70 μ was used for preparative chromatography.

HPLC was performed using a Beckman System Gold chromatographic system on a Beckman Ultrasphere C-18 (4.6 x 150 mm) column with mixtures of acetonitrile-water containing 0.1% trifluoroacetic acid. For preparative runs a Beckman Ultraprep C-18 (10 μ , 21.2 x 150 mm) column was used. Chromatograms were monitored at 214 nm.

2-Trimethylsilylethyl 4-O- β -D-galactopyranosyl- β -D-glucopyranoside (2)

2-Trimethylsilylethyl 2,3,6-tri-O-acetyl-4-O-(2,3,4,6-tetra-O-acetyl- β -D-galactopyranosyl)- β -D-glucopyranoside (1) (Jansson et al., 1988) (29.4 g, 40 mmol) was stirred in methanolic sodium methoxide (5.8 mM, 515 mL) for 15 h. Neutralization with acetic acid, evaporation and crystallization from ethanol gave (2) (14.6 g, 82%).

m.p. 175-177°C

$[\alpha]_D^{22}$: +18.5° (c 1.0, water)

$^1\text{H-NMR}$ (D_2O , $(\text{CH}_3)_3\text{Si}(\text{CD}_2)_2\text{COONa}$) δ : 4.51 (d, 1H, $J=8$ Hz, H-1/H-1'), 4.46 (d, 1H, $J=7.5$ Hz, H-1/H-1'), 4.10-3.50 (13 H), 3.30 (t, 1H, $J=8.5$ Hz), 1.09 (td, 1H, $J=13$, 13 and 6 Hz, CH_2Si), 0.98 (td, 1H, $J=13$, 13 and 5.5 Hz, CH_2Si), 0.04 (s, $(\text{CH}_3)_3\text{Si}$) ppm.

$^{13}\text{C-NMR}$ (D_2O , $(\text{CH}_3)_3\text{Si}(\text{CD}_2)_2\text{COONa}$) δ : 105.7 (C-1/C-1'), 104.2 (C-1/C-1'), 81.2, 78.2, 77.6, 77.4, 75.7, 75.4, 73.8, 71.4, 71.2, 63.8, 62.9, 20.4 (CH_2Si), 0.4 ($(\text{CH}_3)_3\text{Si}$) ppm.

2-Trimethylsilylethyl 2,3,6-tri-O-benzyl-4-O-(2,6-di-O-benzyl-3,4-O-isopropylidene- β -D-galactopyranosyl)- β -D-glucopyranoside (3)

To compound 2 (14.5 g, 32.9 mmol) in 2,2-dimethoxypropane (250 mL) was added p-toluenesulphonic acid monohydrate (1.6 g). The mixture was stirred for 165 min. Triethyl amine (9 mL) was then added and the mixture was evaporated. The residue (containing a mixture of acetals) was treated with aqueous 5% acetic acid (100 mL) for 50 min and was then evaporated to give, according to TLC, one major product and traces of a second component (presumably the 4',6'-acetal).

A part (11.9 g, 25.3 mmol) of the crude product 15.5 g) was dissolved in dry N,N-dimethylformamide under nitrogen. Sodium hydride (60%, 6.16 g, 154 mmol) was added and the mixture was stirred for 1 h. Benzyl bromide (21 mL, 177 mmol) was then added during 15 min. and the stirring was continued for 14 h. The mixture was worked up by addition of methanol followed by water and was then partitioned between dichloromethane and water. The aqueous phase was extracted once with dichloromethane and the combined organic phases were dried (Na_2SO_4), filtered and evaporated. Chromatography (toluene-ethyl acetate) gave compound 3 (15 g, 73%) as a syrup.
[α] D^{22} : +16.5° (c 1.2, chloroform)

$^1\text{H-NMR}$ δ : 7.45-7.25 (25H, Ar-H), 5.0-4.3 (12H, anomeric and benzylic signals), 4.15-3.90 (4H), 3.85-3.30 (10H), 1.42 (s, 3H, CH_3), 1.37 (s, 3H, CH_3), 1.14-0.97 (m, 2H, CH_2Si), 0.05 (s, $(\text{CH}_3)_3\text{Si}$) ppm.
 $^{13}\text{C-NMR}$ δ : 139.3, 139.0, 138.8, 138.7, 138.6, 128.5-127.4, 109.9 ($(\text{CH}_3\text{O})_2\text{C}$), 103.3 (C-1/C-1'), 102.0 (C-1/C-1'), 83.1, 82.0, 80.7, 79.9, 75.4, 75.1, 74.9, 73.6, 73.4, 73.2, 72.0, 68.9, 68.34, 67.3, 27.8, 26.2, 18.3 (CH_2Si), -1.7 ($(\text{CH}_3)_3\text{Si}$) ppm.

2-Trimethylsilylethyl 2,3,6-tri-O-benzyl-4-O-(2,6-di-O-benzyl- β -D-galactopyranosyl)- β -D-glucopyranoside (4)

Compound 3 (14.9 g, 15.9 mmol) in 80% aqueous acetic acid (110 mL) was stirred at 80°C for 35 min. and was then evaporated. Chromatography (toluene-ethyl acetate, 4:1) gave compound 4 (12.3 g, 86%). An analytical sample was crystallized from heptane.
m.p. 98.8-100.1°C
[α] D^{22} : +15.6° (c 1.4, chloroform)

$^1\text{H-NMR}$ δ : 7.45-7.20 (25H, Ar-H), 5.05-4.56 (7H), 4.50-4.35 (5H), 4.10-3.30 (14H), 2.53 (bs, 1H, OH), 2.45 (bs, 1H, OH), 1.13-0.97 (m, 2H, CH_2Si), 0.04 (s, $(\text{CH}_3)_3\text{Si}$) ppm.

^{13}C -NMR δ : 139.4, 139.0, 138.6, 138.5, 138.2, 128.7-127.4, 103.3 (C-1/C-1'), 102.7 (C-1/C-1'), 82.9, 82.6, 80.1, 76.7, 75.2, 74.1, 74.9, 73.5, 73.46, 73.2, 72.8, 68.8, 68.6, 68.4, 67.4, 18.3 (CH_2Si), -1.7 ($(\text{CH}_3)_3\text{Si}$) ppm

5

2-Trimethylsilylethyl 2,3,6-tri-O-acetyl-4-O-{2,4,6-tri-O-acetyl-3-O-[methyl(5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-glycero- α -D-galacto-2-nonulopyranosyl)onate]- β -D-galactopyranosyl]- β -D-glucopyranoside (6)}

10

Compound 4 (8.8 g, 9.9 mmol), 5 (2.93 g, 4.9 mmol) and powdered molecular sieves (3A, 12 g) in dry acetonitrile (114 mL) was cooled under nitrogen to -23°C . To this mixture was added, with stirring, a solution (50 mL) of dimethyl(methylthio)sulfonium trifluoromethanesulfonate (Marra and Sinaÿ, 1990) (25.7 mmol) acetonitrile (50 mL). The reaction mixture was stirred at -23°C for 2h and was then filtered through celite and partitioned between saturated aqueous sodium hydrogencarbonate and dichloromethane. The aqueous phase was extracted once with dichloromethane and the combined organic phases were dried (Na_2SO_4), filtered and evaporated. Chromatography (toluene-ethyl acetate-methanol, 35:35:1) gave a fraction (1.96 g) containing one major and two minor components. Acetylation in acetic anhydride-pyridine (1:1) for 12 h gave, after evaporation, a crude product which was subjected to hydrogenolysis at 35 psi for 4.5 h in glacial acetic acid using 10% palladium on charcoal as a catalyst (50 mL acetic acid, 0.75 g 10% Pd/C per g of substrate). Filtration, evaporation and chromatography (toluene-ethanol 6:1) gave a major fraction which was acetylated as above. Chromatography (toluene-ethanol, 18:1 $\rightarrow$ 10:1) gave amorphous compound 6 (1.0g, 17.5%).

20

25

$[\alpha]_{\text{D}}^{22}$: -8.8° (c 1.2, chloroform)

30

- ¹H-NMR δ : 5.54 (ddd, 1H, $J=9.5$, 5.0 and 2.5 Hz, H-8''), 5.38 (dd, 1H, $J=9.5$ and 2.8 Hz, H-7''), 5.17 (t, 1H, $J=9.5$ Hz, H-3), 5.08 (d, 1H, $J=10.5$ Hz, NH), 4.97-4.82 (4H, H-2, H-2', H-4', H-4''), 4.62 (d, 1H, $J=8$ Hz, H-1'), 4.54-4.37 (4H, *inter alia* H-1, H-3'), 4.18 (dd, 1H, $J=12$ and 5.5 Hz), 4.1-3.8 (10H), 3.83 (s, CH₃O), 3.65-3.49 (3H), 2.58 (dd, 1H, $J=12.5$ and 4.5 Hz, H-3''ekv.), 2.23, 2.15, 2.07, 2.05, 2.03, 2.02, 2.0 and 1.84 (singlets, 3H each, CH₃CO), 2.06 (s, 6H, 2xCH₃CO), 1.67 (t, 1H, $J=12.5$ Hz, H-3''ax.), 1.01-0.81 (m, 2H, CH₂Si), -0.015 (s, (CH₃)₃Si) ppm.
- ¹³C-NMR δ : 171.2, 170.9 (2C), 170.8, 170.7, 170.5, 170.1, 170.0, 169.94, 169.88, 168.3 (C-1''), 101.1 (C-1/C-1'), 100.0 (C-1/C-1'), 96.8 (C-2''), 76.4, 73.6, 72.5, 72.0, 71.9, 71.4, 70.4, 69.9, 69.3, 67.7, 67.4, 67.3, 66.9, 62.3, 62.2, 61.4, 53.0 (CH₃O), 49.0 (C-5''), 37.2 (C-3''), 23.0, 21.3, 20.7-20.4, 17.7 (CH₂Si), -1.8 ((CH₃)₃Si) ppm.
- Anal. Calcd. for C₄₉H₇₂NO₂₉Si: C, 50.4; H, 6.2; N, 1.2. Found: C, 50.3; H, 6.4; N, 1.2.

- 1,2,3,6-Tetra-O-acetyl-4-O-{2,4,6-tri-O-acetyl-3-[methyl (5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-glycero- α -D-galacto-2-nonulopyranosyl)onate]- β -D-galactopyranosyl]- β -D-glucopyranoside (7)}
- Compound 6 (840 mg, 0.72 mmol) was dissolved in a mixture of toluene (5.6 mL) and acetic anhydride (1.06 mL). To this solution was added a solution (1.45 mL) of borontrifluoride etherate (0.36 mL, 2.91 mmol) in toluene (2.04 mL). The reaction mixture was stirred for 140 min. and was then worked up by partition between dichloromethane and saturated aqueous sodium hydrogencarbonate. The aqueous phase was extracted once with dichloromethane and the combined organic phases were dried (Na₂SO₄), filtered and evaporated to give crude (7) (815 mg).
- Crystallization from methanol gave compound 7 (586 mg). Chromatography (toluene-ethanol 20:1 $\rightarrow$ 10:1) of the mother liquor gave additional (7) (154 mg). Total yield of (7) was 740 mg (92%).

According to NMR, compound 7 contained methanol of crystallization in the molar ratio of 0.5 mole methanol per mole of (7). Less than 5% of the 1- α epimer was present as detected by NMR.

$[\alpha]_D^{22}$: -0.2° (c 1.9, chloroform)

5

$^1\text{H-NMR}$ δ : 5.67 (d, 1H, $J=8.5$ Hz, H-1), 5.51 (ddd, 1H, $J=9.5$, 4.5 and 2.5 Hz, H-8"), 5.40 (dd, 1H, $J=9.5$ and 2.5 Hz, H-7"), 5.22 (t, 1H, $J=9$ Hz, H-3), 5.08 (d, 1H, $J=10.5$ Hz, NH), 5.04 (dd, 1H, $J=9.5$ and 8.5 Hz, H-2), 4.96-4.83 (3H, H-2', H-4' and H-4"), 4.65 (d, 1H, $J=8$ Hz, H-1'), 4.50 (dd, 1H, $J=10.5$ and 3.5 Hz, H-3'), 4.42 (dd, 1H, $J=12.5$ and 2 Hz, H-9"), 4.42 (dd, 1H, $J=12.5$ and <2 Hz, H-6/H-6'), 4.20 (dd, 1H, $J=12$ and 5 Hz, H-9"), 4.08-3.80 (9H), 3.84 (s, CH_3O), 3.75 (ddd, 1H, $J=10$, 5 and 2 Hz, H-5/H-5'), 3.62 (dd, 1H, $J=11$ and 3 Hz, H-6/H-6'), 3.45 (d, 1.5 H, $J=5.5$ Hz, CH_3OH), 2.57 (dd, 1H, $J=12.5$ and 5 Hz, H-3"ekv.), 2.24-1.83 (CH_3CO signals), 1.67 (t, 1H, $J=12.5$ Hz, H-3"ax.), 1.09 (q, 0.5 H, $J=5.5$ Hz, CH_3OH) ppm.

10

15

20

$^{13}\text{C-NMR}$ (measured on chromatographed, methanol free material) δ : 171.2, 170.9, 170.7, 170.66, 170.61, 170.5, 169.94, 169.9, 169.8, 169.2, 168.2, 101.0 (C-1'), 96.8 (C-2"), 91.6 (C-1), 75.7, 73.5, 73.1, 71.9, 71.3, 70.6, 70.4, 69.8, 69.2, 67.7, 67.2, 66.7, 62.0, 61.8, 61.5, 53.2 (CH_3O), 49.0 (C-5"), 37.2 (C-3"), 22.9, 21.3, 20.7, 20.6, 20.5, 20.5, 20.4 ppm.

25

3-Bromo-2-bromomethylprop-1-yl 2,3,6-tri-O-acetyl-4-O-(2,4,6-tri-O-acetyl-3-O-[methyl (5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-glycero- α -D-galacto-2-nonulopyranosyl)onate]- β -D-galactopyranosyl)- β -D-glucopyranoside (8)

30

Compound 7 (0.96 g, 0.86 mmol) and 3-bromo-2-bromomethylpropan-1-ol (Ansari *et al.*, 1987) (1.5 g, 6.5 mmol) were dissolved in dry dichloromethane (30 mL) and was then cooled to 0°C. Borontrifluoride etherate (1.9 mL, 15 mmol) was added and the mixture was stirred at 0°C for 40 min. The cooling bath was removed and stirring was continued for 5 h. The mixture was then diluted with dichloromethane and

washed with ice-cold saturated aqueous sodium hydrogencarbonate. The dichloromethane phase was dried (Na_2SO_4), filtered and evaporated. Chromatography (toluene-ethanol, 20:1→12:1) gave amorphous compound 8 (512 mg, 46%).

5 $[\alpha]_D^{22}$: -0.5 ($\leq$ 2.6, chloroform)

10 $^1\text{H-NMR}$ δ : 5.54 (ddd, 1H, $J=9.5$, 5 and 2.5 Hz, H-8"), 5.40 (dd, 1H, $J=9.5$ and 3 Hz, H-7"), 5.19 (t, 1H, $J=9.5$ Hz, H-3), 5.04 (d, 1H, $J=10$ Hz, NH), 4.97-4.82 (4H), 4.67 (d, 1H, $J=8$ Hz, H-1'), 4.54-4.37 (4H), 4.19 (dd, 1H, $J=12$ and 5.5 Hz, H-9"), 4.10-3.80 (10H), 3.84 (s, CH_3O), 3.66-3.42 (8H), 2.58 (dd, 1H, $J=12.5$ and 4.5 Hz, H-3 ekv.), 2.38-2.22 (m, $\text{CH}(\text{CH}_2\text{Br})_2$), 2.24 (s, 3H), 2.16 (s, 3H), 2.10 (s, 3H), 2.08 (s, 6H), 2.07 (s, 3H), 2.06 (s, 6H), 2.04 (s, 3H), 2.00 (s, 3H), 1.85 (s, 3H), 1.68 (t, 1H, $J=12.5$ Hz, H-3"ax.) ppm.

15 $^{13}\text{C-NMR}$ δ : 171.1, 170.94, 170.9, 170.7, 170.6, 170.6, 170.5, 170.0, 169.97, 169.7, 169.8, 166.2 (C-1"), 101.1, 100.8, 96.8 (C-2"), 76.2, 73.1, 72.7, 72.0, 71.6, 71.3, 70.4, 69.6, 69.2, 69.1, 67.7, 67.2, 66.8, 62.1, 61.4, 53.0 (CH_3O), 47.0 (C-5"), 42.4 ($\text{CH}(\text{CH}_2\text{Br})_2$), 37.2 (C-3"), 32.7 (CH_2Br), 31.6 (CH_2Br), 22.9, 21.3, 20.7, 20.5, 20.4, 20.4 ppm.

20

3-Bromo-2-bromomethylpropyl 4-O-{3-O-[sodium (5-acetamido-3,5-dideoxy-D-glycero- α -D-galacto-2-nonulopyranosyl)onate]- β -D-galactopyranosyl]- β -D-glucopyranoside (9)}

25 Compound 8 (384 mg, 0.30 mmol) was dissolved in methanolic sodium methoxide (0.03M, 60 mL) and stirred over night. Water (100 μL) was added and the mixture was neutralized with silica, filtered and concentrated. Chromatography (chloroform-methanol-water, 65:53:6) gave amorphous compound 9 (189 mg, 74%).

$[\alpha]_D^{22}$: -3.4° ($\leq$ 0.3, methanol)

30

$^1\text{H-NMR}$ (CD_3OD) δ : 4.44 (d, 1H, $J=8$ Hz, H-1/H-1'), 4.31 (d, 1H, $J=8$ Hz, H-1/H-1'), 2.86 (dd, 1H, $J=12.5$ and 3.5 Hz, H-3"ekv., virtually

coupled), 3.43-2.31 (m, 1H, $\text{CH}(\text{CH}_2\text{Br})_2$), 2.02 (s, 3H, NCOCH_3), 1.73 (bt, 1H, $J=12.5$ Hz, H-3''ax., virtually coupled) ppm.
 ^{13}C -NMR (CD_3OD) δ : 175.5, 174.9, 105.1 (C-1/C-1'), 104.6 (C-1/C-1'), 101.1 (C-2''), 54.0 (C-5''), 44.4 ($\text{CH}(\text{CH}_2\text{Br})_2$), 42.1 (C-3''), 33.9 (CH_2Br), 33.7 (CH_2Br), 22.7 (NCOCH_3) ppm.

2-Bromoethyl 4-O- β -D-galactopyranosyl- β -D-glucopyranoside (11)

2-Bromoethyl 4-O-(2,3,4,6-tetra-O-acetyl- β -D-galactopyranosyl)-2,3,6-tri-O-acetyl- β -D-glucopyranoside (0.17 mol) was prepared according to Dahmén *et al.* (1983 b). The crude product was dissolved in methanolic sodium methoxide (0.01M, 1 L) and stirred for 3 h. The mixture was neutralized with silica, filtered and concentrated. Chromatography (dichloromethane-methanol-water, 65:20:3) and crystallisation from ethanol gave compound 11 (37g, 51%).

m.p. 151-152°C

^1H -NMR (D_2O , acetone) δ : 4.57 (d, 1H, $J=8.0$ Hz, H-1), 4.45 (d, 1H, $J=8$ Hz, H-1'), 4.26-4.19 (m, 1H, $\text{OCH}_2\text{CH}_2\text{Br}$), 3.98 (dd, 1H, $J=12.5$ and 1 Hz, H-6/H-6'), 3.93 (d, 1H, $J=3.5$ Hz, H-4'), 3.55 (dd, 1H, $J=10$ and 8 Hz, H-2'), 3.40-3.30 (m, 1H, H-2) ppm.

^{13}C -NMR (D_2O , acetone) δ : 105.7 (C-1'), 104.9 (C-1), 75.5 (C-2'), 73.7 (C-2), 71.3 (C-4'), 63.8 (C-6/C-6'), 62.9 (C-6/C-6'), 33.9 (CH_2Br) ppm.

2-Bromoethyl 4-O-(3,4-O-isopropylidene- β -D-galactopyranosyl)- β -glucopyranoside (12)

A mixture of compound 11 (35 g, 78 mmol) and p-toluenesulfonic acid (1.5 g, 8 mmol) in 2,2-dimethoxypropan (800 mL) was stirred at 50° for 1h and then at room temperature over night. Triethylamine (12 mL, 80 mmol) was added and the mixture was stirred for 30 min, concentrated and evaporated with toluene to remove excess triethylamine. The crude product was dissolved in methanol-water (10:1, 900 mL) and boiled under reflux for 2 h. Saturated aqueous sodium hydrogencarbonate (20 mL) was added and the mixture was concentrated. Flash

chromatography (chloroform-methanol-triethylamine, 10:1:0.01) and crystallisation from ethanol gave compound 12 (32.7 g, 86%).

m.p. 171-172°C

$[\alpha]_D^{22}$: +11.1 ($\pm$ 0.7, methanol).

5

$^1\text{H-NMR}$ (D_2O , acetone) δ : 4.56 (d, 1H, $J=8$ Hz, H-1), 4.49 (d, 1H, $J=8.5$ Hz, H-1'), 4.37 (dd, 1H, $J=5.5$ and 1.5 Hz, H-4'), 3.51 (dd, 1H, $J=8$ and 8.0 Hz, H-2'), 3.36 (dd, 1H, $J=9.0$ and 8 Hz, H-2), 1.55 (s, 3H, CH_3), 1.40 (s, 3H, CH_3) ppm.

10

$^{13}\text{C-NMR}$ (D_2O , acetone) δ : 113.7 ($(\text{CH}_3)_2\text{C}$), 104.9 (C-1/C-1'), 81.4 (C-5), 81.3 (C-3'), 77.5 ($\text{CH}_2\text{CH}_2\text{Br}$), 77.0 (C-3), 76.5 (C-4'), 76.2 (C-5'), 75.5 (C-2/C-2'), 63.5 (C-6/C-6'), 62.8 (C-6/C-6'), 33.8 ($\text{CH}_2\text{CH}_2\text{Br}$), 29.9 (CH_3C), 28.1 (CH_3C) ppm.

15

Anal. Calc. for $\text{C}_{17}\text{H}_{29}\text{BrO}_{11}$: C, 41.7; H, 6.0. Found: C, 41.5; H, 6.0.

2-Bromoethyl 2,6-di-O-benzoyl-4-(6-O-benzoyl- β -D-galactopyranosyl)- β -D-glucopyranoside (13)

20

Compound 12 (9.0 g, 18.4 mmol) was dissolved in a mixture of dry pyridine (60 mL) and dichloromethane (150 mL) and cooled to -50°C . Benzoylchloride (10.5 mL, 90.6 mmol) in dry dichloromethane (150 mL) was added dropwise during 3 h and the mixture was stirred for additional 1 h. Methanol was then added and the solution was concentrated. The residue was dissolved in dichloromethane (500 mL) and washed with aqueous hydrogen chloride (2 M, 250 mL), saturated aqueous sodium hydrogencarbonate (250 mL), saturated aqueous sodium chloride (250 mL), dried (Na_2SO_4) and concentrated. The crude product was dissolved in 80% aqueous acetic acid (250 ml), stirred at 80°C for 30 min. and then concentrated. Chromatography (dichloromethane-methanol, 30:1) gave amorphous compound 13 (4.3 g, 31%).

30

$[\alpha]_D^{22}$: +15.8° ($\pm$ 1.0, chloroform).

$^1\text{H-NMR}$ δ : 8.24-8.02 (m, 15 H, Ar-H), 5.19 (dd, 1H, $J=9.5$ and 8 Hz, H-2), 4.84 (dd, 1H, $J=12.0$ and 1.5 Hz, H-6 /H-6'), 4.66 (dd, 1H, $J=12$ and 3.5 Hz, H-6/H-6'), 4.64 (d, 1H, $J=8$ Hz, H-1), 4.48 (dd, 1H, $J=12.0$ and 6 Hz, H-6/H-6'), 4.37 (dd, 1H, $J=12$ and 9 Hz, H-6/H-6'), 4.35 (d, 1H, $J=8$ Hz, H-1') ppm.

$^{13}\text{C-NMR}$ δ : 166.8, 166.6, 165.5, 104.1 (C-1'), 101.0 (C-1), 81.7, 73.5, 73.2, 73.1, 73.02, 72.96, 70.8, 69.6, 68.9, 64.1 (C-6/C-6'), 63.9 (C-6/6'), 29.6 ($\text{OCH}_2\text{CH}_2\text{Br}$) ppm.

Anal. Calc. for $\text{C}_{35}\text{H}_{37}\text{BrO}_{14}$: C, 55.2; H, 4.9. Found: C, 55.2; H, 4.9.

2-Bromoethyl 2,6-di-O-benzoyl-4-O-(6-O-benzoyl-3-O-[methyl (5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-glycero- α -D-galacto-2-nonulopyranosyl)onate]- β -D-galactopyranosyl)- β -D-glucopyranoside (14)

Compound 13 (200 mg, 0.26 mmol), 5 (263 mg, 0.44 mmol) and powdered molecular sieves (3A, 300 mg) in a mixture of acetonitrile and dichloromethane (9:4, 8 mL) was stirred for 1h under argon. Silver trifluoromethanesulfonate (118 mg, 0.45 mmol) was added and the reaction mixture was cooled to -60°C . Methylsulphenyl bromide (Dasgupta and Garegg, 1988) in dichloromethane (3.48 M, 126 μL , 0.44 mmol) was added dropwise during 5 min. and the mixture was then stirred for 1h. Diisopropylamine (0.7 mL, 2.6 mmol) was added and stirring was continued for 0.5 h. The mixture was allowed to attain 0°C , filtered and concentrated. Chromatography (chloroform-methanol, 50:1) gave amorphous compound 14 (244 mg, 75%).

$[\alpha]_{\text{D}}^{22}$: $+14.9^\circ$ (c 1.0, chloroform)

$^1\text{H-NMR}$ δ : 5.33 (m, 1H, H-8''), 5.28 (dd, 1H, $J=8.0$ Hz, H-7''), 5.26 (dd, 1H, $J=9.5$ Hz, H-2), 5.03-4.93 (m, 2H, H-4'', H-6/H-6'), 4.74 (dd, 1H, $J=12$ and 3.5 Hz, H-6/H-6'), 4.70 (d, 1H, $J=8$ Hz, H-1), 4.59 (d, 1H, $J=8$ Hz, H-1'), 4.50 (dd, 1H, $J=12$ and 6 Hz, H-6/H-6'), 2.70 (dd, 1H, $J=13.0$ and 4.5 Hz, H-3''ekv.) ppm.

(H-3, H-2' and H-4' were shifted downfield after acetylation of 14)

^{13}C -NMR δ : 170.7, 170.4, 170.3, 170.1, 170.1, 168.1 (C-1'', JC-1''-H-3'' ax = 6.1 Hz), 166.6, 166.1, 165.4, 104.4 (C-1'), 101.1 (C-1), 97.6 (C-2''), 82.2, 76.4, 73.4, 73.0, 72.9, 63.7, 63.4, 62.4 (C-6, C-6', C-6''), 53.2 (CH₃O), 49.6 (C-5''), 37.6 (C-3''), 29.6 (OCH₂CH₂Br), 23.1 (NCOCH₃), 21.0, 20.7, 20.6, 20.5 ppm.

Anal. Calc for C₅₅H₆₄BrNO₂₆: C, 53.5; H, 5.2. Found: C, 53.5; H, 5.5.

10 2-Bromoethyl 4-O-{3-O-[sodium (5-acetamido-3,5-dideoxy-D-glycero- α -D-galacto-2-nonulopyranosyl)onate]- β -D-galactopyranosyl]- β -D-glucopyranoside (15)}

Compound 14 (200 mg, 0.16 mmol) was dissolved in methanolic sodium methoxide (0.03M, 50 mL) and stirred for 6 h. Water (50 μ L) was added and the mixture was stirred for 5 min., neutralized with silica, filtered and concentrated. Chromatography (chloroform-methanol-water, 6:4:1) gave amorphous compound 15 (118 mg, 96%).
 $[\alpha]_{\text{D}}^{22}$: -0.9 (c 1.0, water)

20 ^1H -NMR (D₂O, acetone) δ : 4.57 (d, 1H, J=8Hz, H-1/H-1'), 4.54 (d, 1H, J 8.5 Hz, H-1 or H-1'), 2.77 (dd, 1H, J 12.2 and 4.3 Hz, H-3e''), 2.03 (s, 3H, NCOCH₃), 1.81 (bt, 1H, H-3a'').

^{13}C -NMR (D₂O, acetone) δ : 177.0, 176.7, 105.6 (C-1'), 105.1 (C-1), 102.8 (C-2''), 54.7 (C-5''), 42.7 (C-3''), 34.0 (OCH₂CH₂Br), 25.0

25 (NCOCH₃) ppm.

2-bromoethyl 4-O-(α -D-galactopyranosyl)- β -D-galactopyranoside (28)

2-Bromoethyl 2,3,6-tri-O-acetyl-4-O-(2,3,4,6-tetra-O-acetyl- α -D-galactopyranosyl)- β -D-galactopyranoside (27) (Dahmén et al. (1983 a) p. 116) was prepared by boron trifluoride etherate catalyzed glycosidation of the corresponding octaacetate (Dahmén et al. (1983 a) p. 113) (1.25 g, anomeric mixture). The crude product was suspended in methanol (20

mL) and methanolic sodium methoxide (0.2 M, 4 mL) was added. A clear solution was obtained after 30 min. The reaction mixture was stirred for additional 30 min. and was then neutralized using silica. Filtration, evaporation and chromatography (chloroform-methanol-water; 65:35:6) gave compound 28 (335 mg, 41%).

[α]_D²²: +69° (c 1.0, methanol)

¹H-NMR (CD₃OD) δ : inter alia 4.76 (d, 1H, J=2 Hz, H-1'), 4.15 (d, 1H, J=7 Hz, H-1), 4.09 (bt, 1H, J=6 Hz), 3.92 (dt, J=11.5 and 6.5 Hz) ppm.

¹³C-NMR (CD₃OD) δ : 105.2, 102.5, 79.2, 76.2, 74.6, 72.7, 71.3, 71.1, 70.7, 62.7, 61.1, 30.9 ppm.
¹³C-NMR (D₂O, ref. acetone at 33.19 ppm): δ : 105.9, 103.2, 80.0, 78.1, 75.2, 73.8, 73.7, 73.1, 72.1, 72.0, 71.6, 63.5, 63.0, 34.1 ppm.

Anal. Calc. for C₁₄H₂₅BrO₁₁: C, 37.4; H, 5.6. Found: C, 37.1; H, 5.9.

2-Bromoethyl 4-O-(4-O- α -D-galactopyranosyl- β -D-galactopyranosyl) β -D-glucopyranoside (48)

2-Bromoethyl 2,3,6-tri-O-acetyl-4-O-[2,3,6-tri-O-acetyl-4-O-(2,3,4,6-tetra-O-acetyl- α -D-galactopyranosyl)- β -D-galactopyranosyl]- β -D-glucopyranoside (53) (Dahmen *et al.* 1984) 35 mg) was deacetylated by stirring in 10 mL of 0.1 M methanolic sodium methoxide for 16 h. Neutralization using silica gel, filtration, evaporation and chromatography (chloroform-methanol-water; 65:35:6) followed by microfiltration and lyophilization gave 48 (18.6 mg, 90%)

[α]_D²²: +51° (c 1.0, water)

¹H-NMR (D₂O, ref. acetone at 2.24 ppm) δ : 4.96 (d, 1H, J=4 Hz, H-1"), 4.57 (d, 1H, J=8.0 Hz, H-1/H-1'), 4.52 (d, 1H, J=7.5 Hz, H-1/H-1), 4.37

(bt, 1H) 4.27-4.19 (m, 1H, $\text{CH}_2\text{CH}_2\text{Br}$) 4.09-3.55 (19H), 3.36 (bt, 1H) ppm

5 ^{13}C -NMR (D_2O , ref. acetone at 33.19 ppm) δ : 106.2 (C-1/C-1'), 105.1 (C-1/C-1'), 103.3 (C-1''), 81.6, 80.3, 78.4, 77.8, 77.3, 75.7, 75.1, 73.9, 73.8, 73.0, 72.1, 71.9, 71.5, 63.5, 63.3, 63.0, 33.9 (CH_2Br) ppm.

2-Bromoethyl glycoside of "GM₃ Lactam" (55)

10 The corresponding decaacetate of the title compound (Ray *et al.* 1992) (40 mg, 1 α /1 β 1:2.2) was de-O-acetylated in 9.5 mM methanolic sodium methoxide at room temperature for 7 h. Neutralization with silica gel, evaporation and chromatography (chloroform-methanol-water, 65:35:6) gave 55 (26 mg. quant.).

15 ^1H -NMR (D_2O , $(\text{CH}_3)_3\text{Si}(\text{CD}_2)_2\text{COONa}$) δ : *inter alia* 3.37 (bt, 1H), 2.61 (dd, 1H, $J=13$ and 6 Hz, H-3''e), 2.05 (s, 3H, NCOCH_3), 1.71 (dd, 1H, $J=11$ and 13 Hz, H-3''a) ppm.

20 The peptides O-glycosylated at serine side chain were synthesised using solid phase peptide synthesis technique. Glycosylation of serine (protected as a 9-fluorenylmethoxycarbonyl- and pentafluorophenyl derivative at the amino- and carboxy groups respectively) with O-acylated β -thioglycosides of Gal α 4Gal gave O-glycosylated serine derivatives. These derivatives were used in solid phase peptide synthesis. After

25 cleavage from the resin and purification by HPLC, glycopeptides were obtained which were still protected at the carbohydrate moiety as acyl derivatives. Complete de-protections were performed using base catalysis, and the products purified by HPLC. FAB-MS data of the products were in agreement with theoretical values.

<u>SEQ ID NO:</u>	<u>Peptides used for conjugation are:</u>	<u>Comp No.</u>	
4	KIASNENMDAMESSTLE C	(16)	(D ^b -restricted)
5	KIASNENMETME	(17)	(D ^b -restricted)
6	KASNENMETMC	(18)	(D ^b -restricted)
7	CASNENMETM	(19)	(D ^b -restricted)
8	CSGPSNTPPEI	(20)	(D ^b -restricted)
9	SGVENPGGYCLT	(26)	(D ^b -restricted)
21	ASNENhETM	(32)	(D ^b -restricted)
22	ASNhNMETM	(33)	(D ^b -restricted)
23	SGPSNhPPEI	(34)	(D ^b -restricted)
24	SGPhNTPPEI	(35)	(D ^b -restricted)
13	CRGYVYQGL	(36)	(K ^b -restricted)
14	CAPGNYPAL	(37)	(K ^b -restricted)
25	RGYhYQGL	(38)	(K ^b -restricted)
26	FAPGhYPAL	(39)	(K ^b -restricted)

<u>SEQ ID NO:</u>	<u>Other examples of peptides suitable for conjugation are:</u>	
10	KQIASNENMETMES C	(D ^b -restricted)
11	KGPSNTPPEI C	(D ^b -restricted)
12	KSGPSNTPPEIHC	(D ^b -restricted)
3	GILGFVFTL	(HLA-A2-restricted)
15	MVVKLGEFYNQMM	(HLA-A2-restricted)
16	CPTNQVVLEGTNKT D	(HLA-A2-restricted)
17	MQIRGFVYFVET	(HLA-A2-restricted)
18	LSPGMMMGMFNM	(HLA-A2-restricted)

(h = homocysteine)

Methods used for the preparation of glycopeptidesMethod A: Conjugation via 2-bromomethylprop-2-en-1-yl spacer

5 A slurry of the 3-bromo-2-bromomethylprop-1-yl glycoside (10 μ mol) and N,N,N,N-tetrabutylammonium fluoride (40-100 μ mol) in N,N-dimethylformamide (100 μ L) was stirred for 10 min. under argon. The peptide (8 μ mol) dissolved in N,N-dimethylformamide (DMF) or dimethylsulfoxide (DMSO) (200-1000 μ L) was added and the mixture was sonicated for 5 min. and then stirred for 1.5-2.5 h. The reaction was
10 monitored by HPLC. The mixture was diluted with 0.1% aqueous trifluoroacetic acid and lyophilized. Preparative HPLC (acetonitrile-water-0.1% trifluoroacetic acid) gave the expected glycopeptides (see Table 2).

Method B: Conjugation via 2-bromoethyl spacer

15 The peptide (5 μ mol) was added to a slurry of the 2-bromoethyl glycoside (10 μ mol) and cesium carbonate (20-100 μ mol) in N,N-dimethylformamide (400 μ L) under argon. The mixture was sonicated for 5 min. and then stirred for 1-5 h. The reaction was monitored by HPLC, diluted with 0.1% aqueous trifluoroacetic acid and lyophilized. Preparative
20 HPLC (acetonitrile-water-0.1% trifluoroacetic acid) gave the expected glycopeptides (see Table 2).

The peptides and the synthesized glycopeptides are present as trifluoromethanesulfonate salts.

25

In addition to the method using cesium carbonate as a promoter, the peptide conjugate 24 was also prepared according to the method of Or et al., HPLC analyses of both the reaction mixtures revealed a major product peak having the same retention time. However, a cleaner
30 reaction and a more easily purified product was obtained with cesium carbonate as a promoter.

Preparation of glycopeptides, examples:Conjugate 21

5 A slurry of 9 (2.9 mg, 3.4 μ mol) and N,N,N,N-tetrabutylammonium fluoride (9.8 mg, 30.6 μ mol) in N,N-dimethylformamide (35 μ L) was stirred for 10 min. under argon. Peptide 16 (8.1 mg, 2.7 μ mol) dissolved in dimethylsulfoxide (350 μ L) was added and the mixture was sonicated for 5 min and then stirred for 1.5 h. The reaction was monitored by HPLC (gradient from 0 to 40% acetonitrile in 0.1% aqueous trifluoroacetic acid, 10 20 min). The mixture was diluted with 0.1% aqueous trifluoroacetic acid and lyophilized. Preparative HPLC (17% acetonitrile in 0.1% aqueous trifluoroacetic acid) gave compound 21 (1 mg, 11%).

15 Retention times (analytical runs): Allylic bromide (from 9): 10.7 min.; 21: 14.2 min.; 16: 15.8 min.

Conjugate 22

20 A slurry of 9 (11 mg, 12.9 μ mol) and N,N,N,N-tetrabutylammonium fluoride (37 mg, 116 μ mol) in N,N-dimethylformamide (130 μ L) was stirred for 10 min under argon. Peptide 17 (20 mg, 10.2 μ mol) dissolved in dimethylsulfoxide (750 μ L) was added and the mixture was sonicated for 5 min and then stirred for 2.5 h. The reaction was monitored by HPLC (gradient from 0 to 40% acetonitrile in 0.1% aqueous trifluoroacetic acid, 20 min). The mixture was diluted with 0.1% aqueous trifluoroacetic acid, 25 and lyophilized. Preparative HPLC (14% acetonitrile in 0.1% aqueous trifluoroacetic acid) gave compound 22 (3 mg, 10%).

Retention times (analytical runs): Allylic bromide (from 9): 10.7 min.; 22: 13.0 min.; 17: 14.3 min.

Conjugate 23

Compound 9 (13 mg, 15.5 μ mol) was treated with N,N,N,N-tetrabutylammonium fluoride (39 mg, 124.0 μ mol) in N,N-dimethylformamide (150 μ L) and peptide 18 (20 mg, 12.0 μ mol) in dimethylsulfoxide (600 μ L) was added and the mixture was sonicated for 5 min and then stirred for 2.5 h. The reaction was monitored by HPLC (gradient from 0 to 40% acetonitrile in 0.1% aqueous trifluoroacetic acid, 20 min). The mixture was diluted with 0.1% aqueous trifluoroacetic acid and lyophilized. Preparative HPLC (12% acetonitrile in 0.1% aqueous trifluoroacetic acid) gave compound 23 (8 mg, 29%).

Retention times (analytical runs): 23: 12.2 min; 18: 13.5 min

Conjugate 24

Peptide 19 (10 mg, 6.8 μ mol) was added to a slurry of compound 15 (10 mg, 13.6 μ mol) and cesium carbonate (11.5 mg, 35.2 μ mol) in N,N-dimethylformamide (550 μ L) under argon. The mixture was sonicated for 5 min and then stirred for 5 h. The reaction was monitored by HPLC (gradient from 0 to 30% acetonitrile in 0.1% aqueous trifluoroacetic acid, 30 min). The mixture was diluted with 0.1% aqueous trifluoroacetic acid and lyophilized. Preparative HPLC (gradient from 12 to 15% acetonitrile in 0.1% aqueous trifluoroacetic acid, 30 min) gave 24 (8.5 mg, 59%).

Retention times (analytical runs): 15: 8.7 min; 24: 18 min; 19: 19.3 min.

Preparation of conjugate 24 according to the method of Or *et al.* (1991):

Compound 15 (8 mg, 10.5 μ mol) and peptide 19 (2 mg, 1.4 μ mol) were dissolved in dry N,N-dimethylformamide (400 μ L) under argon. The solution was saturated with ammonia and stirred for 1.5 h. The reaction was monitored by HPLC (gradient from 0 to 30% acetonitrile in 0.1% aqueous trifluoroacetic acid, 30 min) and showed a major peak (52%)

with the same retention time (18 min) as compound 24 prepared according to method B.

Conjugate 25

5 Peptide 20 (10 mg, 6.0 μ mol) was added to a slurry of compound 15 (9 mg, 12.0 μ mol) and cesium carbonate (39 mg, 120 μ mol) in N,N-dimethylformamide (500 μ L) under argon. The mixture was sonicated for 5 min and then stirred for 1 h. The reaction was monitored by HPLC (gradient from 0 to 30% acetonitrile in 0.1% aqueous trifluoroacetic acid, 30 min) The mixture was diluted with 0.1% aqueous trifluoroacetic acid and lyophilized. Preparative HPLC (gradient from 13 to 18% acetonitrile in 0.1% aqueous trifluoroacetic acid, 30 min) gave compound 25 (14 mg, 60%).

15 Retention times (analytical runs): 15: 8.7 min; 25: 20.5 min; 20: 21.7 min.

Conjugate 29

20 The peptide 20 (20.5 mg, 12.3 μ mol) was added to a slurry of compound 28 (31.6 mg, 70.3 μ mol) and cesium carbonate (110.3 mg, 338 μ mol) in N,N-dimethylformamide (1.6 mL) under argon. The mixture was sonicated for 5 min. and then stirred for 3 h. The reaction was monitored by HPLC (gradient from 0 to 30% acetonitrile in 0.1% aqueous trifluoroacetic acid, 30 min), diluted with 0.1% aqueous trifluoroacetic acid and lyophilized. Preparative HPLC (gradient from 13 to 18% acetonitrile in 0.1% aqueous trifluoroacetic acid, 30 min) gave compound 29 (17.6 mg, 70%).

25 Retention times (analytical runs): 28: 6.2 min, 29: 20.8 min, 20: 21.8 min.

Conjugate 30

30 Peptide 26 (10 mg, 6.5 μ mol) was dissolved in N,N-dimethylformamide (800 mL) under argon. Compound 28 (11.7 mg, 26 μ mol) and cesium carbonate (21.2 mg, 65 μ mol) were added. The mixture was sonicated

for 5 min and then stirred for 2 h. The reaction was monitored by HPLC (gradient from 0 to 30% acetonitrile in 0.1% aqueous trifluoroacetic acid, 30 min). The mixture was diluted with 0.1% aqueous trifluoroacetic acid and lyophilized. Preparative HPLC (gradient from 13 to 18% acetonitrile in 0.1% aqueous trifluoroacetic acid, 30 min) gave compound 30 (11.2 mg, 90%).

Retention times (analytical runs, gradient from 0 to 30% acetonitrile in 0.1% aqueous trifluoroacetic acid, 50 min): 28: 6.2 min, 30: 21.5 min, 26: 24.4 min.

Conjugate 31

Peptide 19 (15 mg, 10 μ mol) was dissolved in N,N-dimethyl formamide (1.2 mL) under argon. Compound 28 (18 mg, 40 μ mol) and cesium carbonate (76.2 mg, 234 μ mol) was added. The mixture was sonicated for 5 min and then stirred for 3 h. The reaction was monitored by HPLC (gradient from 0 to 30% acetonitrile in 0.1% aqueous trifluoroacetic acid, 30 min). The mixture was diluted with 0.1% aqueous trifluoroacetic acid and lyophilized. Preparative HPLC (gradient from 13 to 18% acetonitrile in 0.1% aqueous trifluoroacetic acid, 30 min) gave compound 31 (13.8 mg, 75%).

Retention times (analytical runs): 28: 6.2 min, 31: 17.7 min, 19: 19.1 min.

Conjugate 40

Peptide 38 (10 mg, 7.6 μ mol) was dissolved in N,N-dimethyl-formamide (0.8 mL) under Argon. Compound 28 (13.7 mg, 30.5 μ mol) and cesium carbonate (24.8 mg, 76 μ mol) was added. The mixture was sonicated for 30 min and then stirred for 1 h. The reaction was monitored by HPLC (gradient from 0 to 40% acetonitrile in 0.1% aqueous trifluoroacetic acid, 30 min). The mixture was diluted with 0.1% aqueous trifluoroacetic acid (16 mL) and lyophilized. Preparative HPLC (gradient

from 16 to 21% acetonitrile in 0.1% aqueous trifluoroacetic acid, 30 min) gave 40 (16.6 mg, 52%).

Retention times (analytical runs): 28: 6.7 min, 40: 18.8 min,
5 38: 21 min.

Conjugate 41

Peptide 39 (10 mg, 8 μ mol) was dissolved in N,N-dimethylformamide (0.8 mL) under Argon. Compound 28 (14.3 mg, 32 μ mol) and cesium carbonate (27.6 mg, 85 μ mol) was added. The mixture was sonicated
10 for 15 min and then stirred for 65 min. The reaction was monitored by HPLC (gradient from 0 to 40% acetonitrile in 0.1% aqueous trifluoroacetic acid, 30 min). The mixture was diluted with 0.1% aqueous trifluoroacetic acid (16 mL) and lyophilized. The crude product was pooled with two
15 additional batches prepared analogously from 5 and 6 mg of peptide respectively. Preparative HPLC of the pooled material (gradient from 19 to 24% acetonitrile in 0.1% aqueous trifluoroacetic acid, 30 min) gave 41 (23.7 mg, 85%).

Retention time (analytical runs): 28: 6.7 min, 41: 22.2 min,
20 39: 24.3 min.

Conjugate 42

Peptide 36 (5 mg, 3.6 μ mol) was dissolved in N,N-dimethylformamide (0.4 mL) under Argon. Compound 28 (6.4 mg, 14.3 μ mol) and cesium carbonate (11.6 mg, 35.7 μ mol) was added. The mixture was sonicated
25 for 5 min and then stirred for 2 h. The reaction was monitored by HPLC (gradient from 17 to 22% acetonitrile in 0.1% aqueous trifluoroacetic acid, 30 min). The mixture was diluted with 0.1% aqueous trifluoroacetic acid (8 mL) and lyophilized. Preparative HPLC (gradient from 17 to 22%
30 acetonitrile in 0.1% aqueous trifluoroacetic acid, 30 min) gave 42 (7.1 mg, 95%, purity: 85%).

Retention times (analytical runs): 28: 6.2 min, 42: 26.4 min, 36: 27.6 min.

Conjugate 43

5 Peptide 37 (10 mg, 7.35 μ mol) was dissolved in N,N-dimethyl- formide (0.8 mL) under Argon. Compound 28 (13.2 mg, 29.4 μ mol) and cesium carbonate (23.9 mg, 73.4 μ mol) was added. The mixture was monitored by HPLC (gradient from 5 to 35% acetonitrile in 0.1% aqueous trifluoroacetic acid, 30 min). The mixture was diluted with 0.1% aqueous trifluoroacetic acid (16 mL) and lyophilized. Preparative HPLC (gradient from 17 to 22% acetonitrile in 0.1% aqueous trifluoroacetic acid, 30 min) gave 43 (9.3 mg, 73%).

Retention times (analytical runs): 28: 6.2 min, 43: 24.6 min, 37: 25.9 min.

15 Conjugate 44

Peptide 32 (10 mg, 8 μ mol) was dissolved in dimethylsulfoxide (0.8 mL) under Argon. Compound 28 (14.5 mg, 32 μ mol) was cesium carbonate (26 mg, 80 μ mol) was added. The mixture was sonicated for 10 min and then stirred for 1 h. The reaction was monitored by HPLC (gradient from 0 to 30% acetonitrile in 0.1% aqueous trifluoroacetic acid, 30 min). The mixture was diluted with 0.1% aqueous trifluoroacetic acid (16 mL) and lyophilized. The crude product was pooled with an additional batch prepared analogously from 10 mg of peptide. Preparative HPLC of the pooled material (gradient from 7 to 12% acetonitrile in 0.1% aqueous trifluoroacetic acid, 30 min) gave 44 (14 mg, 54%).

Retention times (analytical runs): 28: 6.2 min, 42: 14.6 min, 32: 16.8 min.

30 Conjugate 45

Peptide 34 (10 mg, 6.8 μ mol) was dissolved in N,N-dimethyl- formamide (0.8 mL) under Argon. Compound 28 (11.2 mg, 25 μ mol) and cesium

carbonate (20.2 mg, 62 μ mol) was added. The mixture was sonicated for 10 min and then stirred for 2 h. The reaction was monitored by HPLC (gradient from 0 to 30% acetonitrile in 0.1% aqueous trifluoroacetic acid, 30 min). The mixture was diluted with 0.1% aqueous trifluoroacetic acid (16 mL) and lyophilized. Preparative HPLC (gradient from 13 to 18% acetonitrile in 0.1% aqueous trifluoroacetic acid, 30 min) gave 45 (7.9 mg, 63%).

Retention times (analytical runs): 28: 6.2 min, 45: 20.5 min, 34: 25.2 min.

Conjugate 46

Peptide 35 (10 mg, 6.7 μ mol) was dissolved in N,N-dimethylformamide (0.8 mL) under Argon. Compound 28 (12.1 mg, 27 μ mol) and cesium carbonate (43.7 mg, 134 μ mol) was added. The mixture was sonicated for 20 min and then stirred for 4 h. The reaction was monitored by HPLC (gradient from 0 to 30% acetonitrile in 0.1% aqueous trifluoroacetic acid, 30 min). The mixture was diluted with 0.1% aqueous trifluoroacetic acid (16 mL) and lyophilized. Preparative HPLC (gradient from 13 to 18% acetonitrile in 0.1% aqueous trifluoroacetic acid, 30 min) gave 46 (17.4 mg, 56%).

Retention times (analytical runs): 28: 6.2 min, 46: 21.0 min, 35: 23.8 min.

Conjugate 47

Peptide 33 (10 mg, 8 μ mol) was dissolved in dimethylsulfoxide (0.8 mL) under Argon. Compound 28 (14.5 mg, 32 μ mol) and cesium carbonate (26 mg, 80 μ mol) was added. The mixture was sonicated for 10 min and then stirred for 1 h 55 min. The reaction was monitored by HPLC (gradient from 0 to 30% acetonitrile in 0.1% aqueous trifluoroacetic acid, 30 min). The mixture was diluted with 0.1% aqueous trifluoroacetic acid (16 mL) and lyophilized. The crude product was pooled with two additional batches prepared analogously from 5 and 10 mg of peptide

respectively. Preparative HPLC of the pooled material (gradient from 10 to 15% acetonitrile in 0.1% aqueous trifluoroacetic acid, 30 min) gave 47 (18.9 mg, 59%).

- 5 Retention times (analytical runs): 28: 6.2 min, 47: 18.3 min, 33: 20.6 min.

Conjugate 49

- 10 Peptide 26 (15 mg, 9.8 μ mol) was dissolved in N,N-dimethylformamide (1.2 mL) under Argon. Compound 48 (23.8 mg, 39 μ mol) and cesium carbonate (31.9 mg, 98 μ mol) was added. The mixture was sonicated for 10 min and then stirred for 1 hr 50 min. The reaction was monitored by HPLC (gradient from 0 to 30% acetonitrile in 0.1% aqueous trifluoroacetic acid, 30 min). The mixture was diluted with 0.1% aqueous trifluoroacetic acid (24 mL) and lyophilized. Preparative HPLC (gradient from 13 to 15 18% acetonitrile in 0.1% aqueous trifluoroacetic acid, 30 min) gave 49 (17.8 mg, 88%).

Retention times (analytical runs): 48: 10.1 min, 49: 21.9 min, 26: 24.4 min.

20

Conjugate 50

- 25 Peptide 32 (15 mg, 21.1 μ mol) was dissolved in a mixture of dimethylsulfoxide (0.6 mL) and N,N-dimethylformamide (0.6 mL) under Argon. The mixture was sonicated for 1 min. Compound 48 (29.6 mg, 48 μ mol) and cesium carbonate (39.4 mg, 121 μ mol) was added. The mixture was sonicated for 15 min and then stirred for 1 h 50 min. The reaction was monitored by HPLC (gradient from 0 to 30% acetonitrile in 0.1% aqueous trifluoroacetic acid, 30 min). The mixture was diluted with 0.1% aqueous trifluoroacetic acid (24 mL) and lyophilized. The crude product was pooled with an additional batch prepared analogously from 5 30 mg of peptide. Preparative HPLC of the pooled material (gradient from 7

to 12% acetonitrile in 0.1% aqueous trifluoroacetic acid, 30 min) gave 50 (14.3 mg, 74%).

Retention times (analytical runs): 48: 10.1 min, 50: 15.2 min, 26: 16.8 min.

5

Conjugate 51

Peptide 19 (15 mg, 10 μ mol) was dissolved in N,N-dimethylformamide (0.4 mL) under Argon. Compound 55 (6 mg, 8.3 μ mol) and cesium carbonate (56 mg, 172 μ mol) was added. The mixture was sonicated for 10 min and then stirred for 2 h. The reaction was monitored by HPLC (gradient from 0 to 30% acetonitrile in 0.1% aqueous trifluoroacetic acid, 30 min). The mixture was diluted with 0.1% aqueous trifluoroacetic acid and lyophilized. Preparative HPLC (gradient from 10 to 14% acetonitrile in 0.1% aqueous trifluoroacetic acid, 30 min) gave 51 (8.2 mg, 45%).

15

Retention times (analytical runs): 55: 13.0 min, 52: 20.3 min, 19: 21.3 min.

Conjugate 52

Peptide 20 (5 mg, 3 μ mol) was dissolved in N,N-dimethylformamide (0.4 mL) under Argon. Compound 55 (4.3 mg, 6 μ mol) and cesium carbonate (37 mg, 114 μ mol) was added. The mixture was sonicated for 5 min and then stirred for 3 h 45 min. The reaction was monitored by HPLC (gradient from 0 to 30% acetonitrile in 0.1% aqueous trifluoroacetic acid, 30 min). The mixture was diluted with 0.1% aqueous trifluoroacetic acid (8 mL) and lyophilized. The crude product was pooled with an additional batch prepared analogously from 5 mg of peptide. Preparative HPLC of the pooled material (gradient from 13 to 18% acetonitrile in 0.1% aqueous trifluoroacetic acid, 30 min) gave 52 (9.3 mg, 65%).

30

Retention times (analytical runs): 55: 13.0 min, 52: 22.7 min, 20: 23.6 min.

TABLE 2

Starting material (Nos.)	Method	Reaction conditions	Isolated product (No.)	Yield (%)	Analysis
9 + 16	A	DMSO, 1000 μ L ^a , 1.5 h	21	11	HPLC ^c
9 + 17	A	DMSO, 750 μ L ^a , 2.5 h	22	10	HPLC ^c
9 + 18	A	DMSO, 500 μ L ^a , 2.5 h	23	29	HPLC ^c , [M+H] ⁺ 1943 ^d
15 + 19	B	DMF, 400 μ L ^b , 5 h	24	59	HPLC ^c , [M+H] ⁺ 1788 ^d
15 + 20	B	DMF, 400 μ L ^b , 1 h	25	60	HPLC ^c , [M+H] ⁺ 1761 ^d
28 + 20	B	DMF, 1300 μ L ^b , 3 h	29	70	HPLC ^c , [M+H] ⁺ 1469 ^d
28 + 26	B	DMF, 615 μ L ^b , 2 h	30	90	HPLC ^c , [M+H] ⁺ 1564 ^d
28 + 19	B	DMF, 600 μ L ^b , 3 h	31	75	HPLC ^c , [M+H] ⁺ 1497 ^d
28 + 38	B	DMF, 525 μ L ^b , 1.5 h	40	52	HPLC ^c , [M+H] ⁺ 1342 ^d
28 + 39	B	DMF, 500 μ L ^b , 1.3 h	41	86	HPLC ^c , [M+H] ⁺ 1320 ^d
28 + 36	B	DMF, 560 μ L ^b , 2.1 h	42	95	HPLC ^c , [M+H] ⁺ 1426 ^d
28 + 37	B	DMF, 545 μ L ^b , 1.75 h	43	73	HPLC ^c , [M+H] ⁺ 1273 ^d

Starting material (Nos.)	Method	Reaction conditions	Isolated product (No.)	Yield (%)	Analysis
28 + 32	B	DMSO, 500 μL^b , 1.2 h	44	54	HPLC ^c , [M+H] ⁺ +1380 ^d
28 + 34	B	DMF, 590 μL^b , 2.2 h	45	63	HPLC ^c , [M+H] ⁺ +1382 ^d
28 + 35	B	DMF, 600 μL^b , 4.3 h	46	56	HPLC ^c , [M+H] ⁺ +1396 ^d
28 + 33	B	DMSO, 500 μL^b , 2.1 h	47	59	HPLC ^c , [M+H] ⁺ +1382 ^d
48 + 26	B	DMF, 612 μL^b , 2 h	49	88	HPLC ^c , [M+H] ⁺ +1726 ^d
48 + 32	B	DMSO + DMF (1:1), 495 μL^b , 2.1 h	50	74	HPLC ^c , [M+H] ⁺ +1542 ^d
55 + 19	B	DMF, 200 μL^b , 2.2 h	51	45	HPLC ^c , [M+H] ⁺ +1769 ^d
55 + 20	B	DMF, 665 μL^b , 3.8 h	52	65	HPLC ^c , [M+H] ⁺ +1741 ^d

^a $\mu\text{L}/10$ mmol peptide.

^b $\mu\text{L}/5$ μmol peptide.

^c The product was in all cases faster moving than the peptide starting material and slower moving than the carbohydrate starting material.

^d FAB-MS data were in agreement with the theoretical values.

2. IN VIVO PRIMARY INDUCTION OF VIRUS-SPECIFIC CTL BY IMMUNIZATION WITH 9-MER SYNTHETIC PEPTIDES

2.1. Methods

5

In vivo primary induction of cytotoxic T lymphocytes (CTL) by immunization with the novel conjugates of the present invention can be carried out by methodology analogous to what is described below for *in vivo* primary induction of virus-specific CTL by immunization with 9-mer synthetic peptides.

10

Vaccination of mice with pre-processed synthetic peptides, corresponding to endogenous 9-mers produced in influenza A virus-infected cells resulted in strong primary CTL responses. The generated CTL efficiently killed virus-infected target cells with preference for viral strains having the identical amino acid sequences to the peptides used for immunization. The optimal conditions for a primary *in vivo* CTL response was obtained with 100 µg peptide dissolved in IFA and injected s.c. at the base of tail. Spleen cells which had been primed 7-10 days earlier were restimulated for 5 days *in vitro*, using an optimal low peptide concentration (0.05 µM) and tested against virus-infected and peptide-treated target cells. The peptide-induced CTL were MHC class I restricted and CD8 positive.

15

20

Peptides

25

The peptide ASNENMETM (designated as pep 9(PR8); SEQ ID NO: 1) and ASNENMDAM (designated as pep 9(NT60); Residue 3-11 of SEQ ID NO: 4) are from the NP366-374 of influenza A virus strains A/PR/8/34 and A/NT/60/68, respectively. Pep 9(PR8) was synthesized using an Applied Biosystems 430A peptide synthesizer (Applied Biosystems, Inc., Foster City, CA). Pep 9(NT60) was synthesized by the solid phase "tea bag" (Houghten, 1985; Sällberg, 1991). All peptides were purified by reverse-phase HPLC and analysed for amino acid composition by plasma

30

desorption mass spectrometry. Stock solutions of peptides were prepared in PBS and stored at -20°C.

Immunization

- 5 Mice were immunized by one s.c. injection of 100 µg each free synthetic peptides dissolved in IFA at the base of the tail or by one intravenous injection of 20 HAU of influenza A virus diluted in PBS and used between 1-2 week after the immunization.

10 In vitro generation of influenza A virus-specific CTL

- Immune spleen cells were prepared into cell suspension. The red cells were lysed using lysis buffer consisting of NH_4Cl 8.29g, KHCO_3 1.0g, EDTA 0.0372g per 1 liter distilled water (pH 7.4). 25×10^6 responder spleen cells from *in vivo* primed mice were cocultured with 25×10^6 irradiated (2000 rad) syngeneic spleen cells, either infected by influenza A virus or in the presence of 0.05, 0.5 and 5 µM peptides in 50-ml tissue culture flask (Costar, Cambridge, MA) with 10 ml complete medium for 5 days at 37°C in humidified air with 5.3% CO_2 .

20 Cytotoxicity assay

- As previously described (Jondal, 1975), virus-infected or peptide-coated target cells were prepared by infecting 2×10^6 cells with 320 HAU virus for 1.5 h or incubated with 50 µM peptides for 1.5 h at 37°C, respectively. After washing, the cells were labelled with 100-200 µCi of $\text{Na}_2^{51}\text{CrO}_4$ for 1 h. The cells were washed twice, and 10^4 target cells in 50 µl were added to 100 µl of varying numbers of effector cells which had been washed three times prior to the assay in 150 µl of complete medium in 96-well V-bottomed plates and were incubated for 4 h at 37°C, 5.3% CO_2 . After incubation, the 50 µl supernatant was collected and the percentage of specific ^{51}Cr release was calculated by the formula: % specific release = (experimental-spontaneous) / (maximal-spontaneous) cpm. Spontaneous release was always less than 15%.

2.2. Results

Primary *in vivo* induction of anti-influenza A virus-specific CTL with 9-mers.

5 As previously reported (Townsend et al., 1985, 1986), CTL induced with live influenza A virus in H-2^b mice are mainly directed against the immunodominant NP365-380 epitope. We initially tried to immunize mice with peptide NP365-380 derived from both PR8 and NT60 strains to induce CTL responses according to a protocol described by Alchele et al.
10 (1990). However, an insignificant level of killing activity against both peptide-coated and virus-infected cells was observed in spite of repeated boosting. Instead, when 100 µg of the shorter pep 9(PR8) was used for s.c. injection and spleen cells restimulated with 5 µM pep 9(PR8) *in vitro*, a strong CTL response against pep 9(PR8)-coated RMA (not shown) and
15 EL4 cells was elicited although virus-infected target cells were not killed (Table 3). When a lower concentration of pep 9(PR8) was used for restimulation *in vitro*, the generated CTL had a higher killing activity against pep 9(PR8)-coated target cells and virus-infected cells were also lysed (Table 3). As shown in Table 3, the optimal dose for immunization
20 was 100 µg and for restimulation 5 or 0.05 µM. Immunization with 10 µg and 1 µg generated less or even no CTL response. Peptide dissolved in complete Freund's adjuvant (CFA) or PBS gave no response. Intravenous injection of peptides did not generate a primary *in vivo* CTL response either (not shown).

25 Another 9-mer derived from NT 60 gave stronger anti-peptide and antiviral CTL responses, as shown in Fig. 2.

Specificity of CTL induced by pep 9(PR8) and pep 9(NT60)

30 In the light of these results we investigated the fine specificity of the generated CTL in relation to pep 9(PR8) and pep (NT60). CTL preferentially killed their corresponding peptide-coated and virus-infected

cells (Fig 4). CTL showed a higher cross-reactivity with peptide-coated cells than with virus-infected target cells.

CTL responses to live virus or peptides

5 Mice were primed with live PR8 virus and immune spleen cells were restimulated with either virus-infected stimulator cells (Fig. 5A) or normal syngeneic spleen cells in the presence of 0.05 μ M pep 9(PR8) (Fig. 5B). CTL primed *in vivo* with live virus and restimulated with low concentration of pep 9(PR8) had a remarkable ability to recognize both virus-infected and peptide-coated cells (Fig 5B). It should be noted that the low amount of restimulation peptide was sufficient to stimulate virus-primed CTL *in vitro* and had the same potency as virus-infected cells did. When pep 9(PR8) was used for *in vivo* priming and virus-infected cells or pep 9(PR8) for restimulation *in vitro* (Fig 5C and D), CTL responses were generated against both peptide-coated and virus-infected cells. Live virus had a higher efficiency to prime CTL than the free peptide.

Time course of CTL activity after immunization with pep 9(PR8)

20 In order to determine the kinetics of CTL activity after immunization with pep 9(PR8), mice were primed once with pep 9(PR8). Spleen cells of immunized animals were restimulated *in vitro* 2-30 days after immunization with pep 9(PR8) and assayed for CTL activity. The CTL activity against pep 9(PR8)-coated RMA target cells reached a peak 7 days after the immunization and the activity gradually declined afterwards (Fig. 6). At the day 30 after priming, a lower level of CTL activity could still be detected.

In vivo peptide-induced cytotoxicity is mediated by CD8⁺ and MHC class I-restricted

30 As shown in Fig. 7a, pep 9(PR8)-induced CTL depleted for CD8⁺ T cells failed to lyse pep 9(PR8)-coated EL4 cells. On the other hand, depletion of CD4⁺ T cells did not affect the cytolytic activity against pep 9(PR8)-

coated target cells. Thus, pep 9(PR8)-specific CTL express the CD4⁺ CD8⁺ phenotype.

In vivo peptide-primed CTL of C57B6/J (H-2^b) origin were tested on syngeneic EL4 (H-2^b), allogeneic P815 (H-2^d) and L929 (H-2^k) target cells infected with influenza PR8 strain. As Fig 7b shows, there is a clear restriction specificity for H-2^b target cells.

TABLE 3

CTL activity induced by different doses of peptides for priming *in vivo* and restimulation *in vitro*.

Priming dose (μg)	Restimulation dose (μM)	Untreated		EL4 target lysis (%)			
		40:1*	20:1	PR8-infected		pep9(PR8)-coated	
				40:1	20:1	40:1	20:1
100	5.00	4.61	4.21	7.00	8.50	43.68	34.73
	0.05	0.12	0.51	30.76	30.07	59.42	50.73
10	5.00	5.63	4.19	15.04	13.98	23.54	22.09
	0.05	5.74	4.14	31.19	23.75	45.52	34.60
1	5.00	3.34	2.66	6.88	5.54	11.85	9.28
	0.05	4.08	3.32	12.43	10.50	16.44	15.54

* Effector:target ratio.

3. EXTERNAL MHC-1 BINDING PEPTIDES ARE RECYCLED TO THE CELL SURFACE AFTER INTERNALIZATION

External peptides that bind MHC-1 are known to upregulate the expression of the corresponding restriction element in the presence of B₂-M (Otten et al., 1992). This upregulation response *in vitro* correlates with the *in vivo* immunogenicity of peptides. As we have found that the endosomal inhibitor chloroquine inhibits this upregulation (Figure 8), it is possible that this process require peptide internalization and membrane recycling. In support of this model a number of earlier studies have found

internalization of MHC-1 through clathrin coated pits, and also MHC-1 recycling.

To study the possible recycling of external MHC-1 binding peptides we synthesized two D^b binding peptides that represented immunodominant epitopes on the E1A protein from adenovirus (sequence SGPSNTPPEI; SEQ ID NO: 2) and on the nucleoprotein from influenza A virus (PR8) (sequence ASNENMETM; SEQ ID NO: 1). Cystein was added to the amino-or carboxytermini, as a biochemical coupling site for the molecular marker galabios (Gal-Gal), for which a specific monoclonal antibody was available (MC2101) (Brodin et al. 1988). As earlier reported, the addition of cystein to the aminotermius did not abrogate the D^b upregulating capacity, or the *in vivo* immunogenicity of the peptide (Zhou et al., 1992 b). The addition of galabios at the same site, generated glycopeptides which were strongly recognized by the galabios specific monoclonal antibody MC2101 in an ELISA test (Table 4). The Gal₂-CSG11 peptide (conjugate 29) reacted stronger than the GAL₂-CAS10 peptide. To verify that the additional galabios did not alter the D^b binding capacity or the *in vivo* immunogenicity, both glycopeptides were tested for *in vitro* D^b upregulation and for *in vivo* immunogenicity. D^b upregulation was similar with glycopeptides, as compared to peptides alone (data not shown). CTL:s generated by *in vivo* injection, and *in vitro* restimulation with glycopeptides were strictly peptide specific and did not recognize Gal₂ when presented by another peptide, in a criss-cross fashion (Figure 9). From these results we conclude that Gal₂, in the glycopeptides, acted as an inert marker.

To allow maximum binding of external glycopeptides to membrane D^b molecules, mutant RMA-S cells were used. These cells are inherently deficient in transporting processed peptides from the cytosol to the ER compartment by the loss of the Tap-2 peptide transporter system. As a consequence, RMA-S cells express a higher fraction of empty D^b

molecules, than non-mutant cells, at the cell surface. The expression of these empty D^b molecules can be further increased by low temperature (26°C), and stabilized by the addition of D^b binding peptides at 37°C (Figure 3). Thus, by treating low temperature induced RMA-S cells with a high concentration of glycopeptides, in the presence of B₂-M, a large fraction of membrane D^b molecules became saturated with the same, identical glycopeptide. RMA-S cells treated this way are clearly stained with the MC2101 antibody (Figure 10), demonstrating membrane expression of the Gal₂ epitope, presumably in the form of MHC-1 bound glycopeptide. By pronase treatment, all D^b and Gal₂ expression is removed from these cells (Figure 10). If these cells are then transferred to 37°C and incubated for 1 hour, both D^b and Gal₂ expression return to the cell surface (Figure 10). The return of the Gal₂ epitope was inhibited by chloroquine (data not shown). As the conventional MHC-1 presentation pathway is non-functional in RMA-S cells, and consequently cytosolic peptides are not transported into the ER compartment, the results strongly indicate endosomal recycling of the D^b binding glycopeptides. Similar results were obtained with the Gal₂-CAS10 glycopeptide (not shown).

To verify these results, on a functional level, influenza A (PR8) specific CTL were generated, and tested against RMA-S and EL-4 target cells treated with a peptide (ASNENMETM) corresponding to the target epitope in the Gal₂-CAS10 glycopeptide (conjugate 31). Both target cells were strongly killed, as earlier reported (Rötzshke et al., 1990) (Figure 11). Pronase treatment of these peptide treated target cells, removed most D^b expression, and most of the sensitivity to the specific CTL:s (Figure 11). Incubation of pronase treated cells at 37°C, to allow re-expression of both D^b and associated peptides, resulted in a return of susceptibility to CTL killing. This return was faster and more extensive with RMA-S cells as compared to EL-4 cells, and was blocked by anti-D^b monoclonal antibodies and by chloroquine (data not shown).

We interpret the present results to mean that MHC-1 bound peptides recycle through an intra-cellular compartment similar to early endosomes in T cells. Possibly this mechanism may allow for peptide exchange to occur in order to optimize the expression of membrane peptides.

5 Hochman et al. (1991) have shown that class I MHC molecules undergo conformational changes in an endosomal compartment, indicating that B₂-M is going off and on the heavy chain. Thus, the low pH that cause this effect, may also allow for peptide exchange to occur. In support of this notion, Harding (1992) has recently reported that MHC-1
10 presentation can be blocked by hypothermia and weak base amines, using electroporation of exogenous antigen.

MHC-1 molecules are known to bind optimal length peptides with a much higher affinity, as compared to slightly longer peptides, a phenomenon
15 that is reflected in a number of *in vitro* assays. These include MHC-1 membrane upregulation, target cell sensitization to specific CTL, direct binding of peptide to empty class I chains on mutant cells, peptide-induced MHC-1 assembly in lysates from mutant cells and
20 measurements of off rates of bound peptides from MHC-1. This higher affinity of optimal length peptides may be related to an exact fit into the peptide binding groove of class I chains, as this groove is closed at both ends (Madden et al., 1991). Thus, the optimally sized peptide may form a trimeric peptide-heavy chain B²-M complex, which is more likely to
25 recycle from endosomes, as compared to complexes consisting of longer peptides.

In target T cells, recycling of optimal MHC-1 bound peptides, may allow for the most efficient recognition by the corresponding, specific CTL:s, as in the present work. So far, most evidence for MHC-1 recycling has
30 been obtained in T cells. However, if similar mechanisms operate in certain antigen presenting cells, the build-up of optimal peptides, at the membrane level, may also be important in the afferent arm of the

immune response. In particular, dendritic cells, known to be crucial for CTL generation, both *in vivo* and *in vitro*, should be investigated in this aspect. The existing correlation between the capacity of peptides to upregulate MHC-1 expression *in vitro*, and their *in vivo* immunogenicity, suggests that this may be an important mechanism in the cellular response.

TABLE 4

Recognition of Gal₂ glycopeptides by monoclonal antibody MC2101 in ELISA assay.

Antibody	Specificity	Absorbance index			
		Gal ₂ -CSG11	CSG-11	Gal ₂ -CAS10	CAS-10
MC2101	Galabios Gal-Gal	1.86	0.01	0.41	0.02
Pk002	Globotriaosyl Gal-Gal-Glc (CD77)	0.04	0.08	0.04	0.01

Peptides (50 µg/ml) were diluted in 0.05 M carbonate buffer (pH 9.6) and 100 µl/well was added to flat-bottomed Costar microplates (cat. No 3590) for incubation over-night at 4°C. Plates were washed once with PBS, pre-incubated with 0.5% PBS for 30 minutes at room temperature and washed twice with PBS/0.05% Tween buffer. Monoclonal antibodies were diluted in 0.5% gelatin/PBS/0.05% Tween, added to microplates and incubated for 2 hours at room temperature, and flick-washed with PBS/Tween. Alkaline phosphatase conjugated rabbit anti-mouse immunoglobulins (Dakopatts code No. S 414) was diluted 1/1000 in PBS/Tween and added as 100 µl/well. After 2 hours incubation at room temperature and 4 washes with PBS/Tween, 100 µl of alkaline

phosphatase substrate solution was added. Plates were read after 2 hour at room temperature, using a Multiskan System (Lab systems), for optical density at 405 nm.

5 4. GENERATION OF A CARBOHYDRATE SPECIFIC CTL RESPONSE BY VACCINATION WITH A GLYCOPEPTIDE WHICH HAS THE CARBOHYDRATE PART IN AN INTERNAL, IMMUNOGENIC POSITION.

10 The carbohydrate galabiose (Gal- α -4Gal otherwise referred to as Gal₂) was coupled to an internal cystein (position 10) in the 12-mer peptide SGVENPGGYCLT (SEQ ID NO: 9) which represents an H2-D^b restricted immunodominant CTL epitope in the lymphocytic choriomeningitis virus (LCMV) (Oldstone et al., 1988). The glycopeptide was called SGV12-Gal₂ (conjugate 30; Figure 12). The coupling was done to a thiol group,
15 using S bonding, as described i Example 1.

Mice were immunized with the SGV12-Gal₂ glycopeptide, as described in Example 2 and in Zhou et al.(1992 a). After restimulation *in vitro* with the same SGV12-Gal₂ glycopeptide, the generated cytotoxic T cells were
20 tested against H2-D^b and K^b positive EL-4 cells, coated with the following peptides and glycopeptides:

1. SGVENPGGYCLT (SGV12; SEQ ID NO: 9)
2. SGV12-Gal₂
- 25 3. ASNENSETM (ASN9; SEQ ID NO: 1) with Gal₂ bound to S in position 6. (ASN9-6S-Gal₂)
4. ASN9-6S
5. CSG11-Gal₂ (see Example 3 and Figure 9)
6. CSGPSNTPPEI (CSG11; SEQ ID NO: 8)
- 30 7. CRG9 with Gal₂ bound to C in position 1 (CRG9-Gal₂)
8. CRGYVYQGL (CRG9; SEQ ID NO: 13)

Peptides 1-6 are known, modified D^b binding T cell epitopes, and bind to RMA-S cells, as measured by D^b upregulation *in vitro* (data not shown). Peptides 7-8 represent a known K^b epitope (RGYVYQGL; SEQ ID NO: 27), and both peptides upregulate K^b on RMA-S cells (data not shown).

5 The expression of the Gal₂ epitope was measured by FACS on the surface of cells that have bound glycopeptides. A high Gal₂ expression was found with glycopeptides 2, 5 and 7 (data not shown). Glycopeptide 3 was not recognized by anti-Gal₂ monoclonal antibodies when bound to RMA-S cells. The conclusion was that glycopeptides 5 and 7 expressed
10 the same Gal₂ epitope as the immunogen SGV12-Gal₂ on different carrier peptides. The CSG11-Gal₂ glycopeptide was bound to D^b and the CRG9-Gal₂ glycopeptide to K^b. Thus, the only epitope that is shared between glycopeptides 2, 5 and 7 is Gal₂.

15 When the SGV12-Gal₂ generated CTL cells were tested against EL-4 cells coated with the above described peptides and glycopeptides, it was found that target cells coated with glycopeptides 2 and 5 were killed, and also, at a lower level, target cells coated with glycopeptide 7 (Figure 13). Thus, the generated CTL cells did not recognize the carrier peptide alone
20 (SGV12), or the other control peptides, including glycopeptide 3 (which does not express the Gal₂ epitope at the cell surface level).

From the above results, it is concluded that immunization with the glycopeptide SGV12-Gal₂, generates a Gal₂ specific T cell response.

25 The reason for this may be that the Gal₂ carbohydrate part is oriented in an optimal position for T cell recognition. The higher killing of glycopeptide 5 coated target cells, as compared to glycopeptide 7 coated target cells, may be due to the fact that glycopeptide 5 is presented by D^b and glycopeptide 7 by K^b class I chains.

30 In a further set of experiments (adopting the same protocol as described above) mice were immunised with glycopeptide RGY8-4h-Gal₂ ("RGY8"

represents the octapeptide RGYVYQGL; 4h indicates that the V in position 4 is replaced by homoserine; Gal₂ indicates conjugation of the sugar Gal₂ to the homoserine hydroxyl). As in the experiments described with reference to Figure 13, the resulting RGY8-4h-Gal₂ generated CTL cells were tested against EL-4 cells coated with RGY8-4h-Gal₂ and RGY8-4h.

From the results shown in Figure 14(A) it can be seen that both peptide and glycopeptide specific CTL cells are generated.

When this heterogeneous effector cell population was tested against EL-4 cells coated with Gal₂ on another carrier peptide (SGV12-Gal₂) and the SGV12 peptide *per se* (Figure 14(B)), only the cells coated with glycopeptide were killed.

This effect proves that the CTLs have Gal₂ specificity by "criss-cross" testing (see the section headed "Experimental Test System" above).

The data presented herein may be summarised in the following Tables 5 and 6.

TABLE 5

Analysis of K^b binding peptides and glycopeptides

Peptide	ELISA	MHC-1 upreg	Membrane express	Immuno- genic	Specificity P G GP
Gal ₂ -CAP9	+	+	—	+	+
FAP9-5h-Gal ₂	+			+	+
Gal ₂ -CRG9	+	—	+	+	+
RGY8-4h-Gal ₂	+	+	+	+	+

Peptide	Immunogenic	Comments
CAP9 (SEQ ID NO: 14)	+	
FAP9 (SEQ ID NO: 20)	+	Strong, with high FAP9-5h reactivity
FAP9-5h (SEQ ID NO: 26)	+	Weaker, low FAP9 reactivity
CRG9 (SEQ ID NO: 13)	+	
RGY8 (SEQ ID NO: 27)	+	Some RGY8-4h reactivity
RGY8-4h (SEQ ID NO: 25)	+	Strong, with RGY8 reactivity

TABLE 6

Analysis of D^b binding peptides and glycopeptides

Analysis of D ⁺ binding peptides and glycopeptides										
Peptide		ELISA 7.4 9.6		MHC-1 upreg	Membrane express recycle		Immuno- genic	Specificity P G GP		
Gal ₂ -CAS10			+	+	+	+	+			+
GM3-CAS10							tox			
<u>GM3-laktam-CAS10</u>							+			+
ASN9-6h-Gal ₂		+	-	+	+	+	-			
ASN9-6S-Gal ₂			-	+		-				
ASN9-4S-Gal ₂		(+)	-			-				
Gal ₂ -CSG11			+	+	+	+	+			+
GM3-CSG11							+			+
<u>GM3-laktam-CSG11</u>							-			
SGP10-6h-Gal ₂		+	+	+	+	+	-			
SGP10-6S-Gal ₂			-	+		-	-			
SGP10-4S-Gal ₂		(+)	-			-				
SGV12-Gal ₂			+	+	+	+	+			+
Peptide		Immunogenic								
CAS10	(SEQ ID NO: 7)	+								
ASN9	(SEQ ID NO: 1)	+								
ASN9-6h	(SEQ ID NO: 21)									
ASN9-6S		-								
<u>ASN9-4S</u>										
CSG11	(SEQ ID NO: 8)	+								
SGP10	(SEQ ID NO: 2)	+								
SGP10-6h	(SEQ ID NO: 23)									
SGP10-6S		+								
SGP10-4S										

Note on Assays (Tables 5 and 6):

ELISA

The ELISA assay was performed as described in connection with

5 Table 4.

MHC-1 upregulation

As described above in relation to Fig 8 and Fig 10A, the principle is that peptides can bind to empty MHC-1 heavy chains at the cell surface on antigen-presenting cells, and this leads to a stabilization, and upregulation of the corresponding MHC-1 chain.

10

Membrane expression

When the glycopeptide bind to MHC-1, on the cell surface, the expression (and accessability) of the corresponding CHO part, can be detected by antibody staining in FACS, as described above in relation to Fig 10C and E.

15

Membrane recycling

Described in Fig 10D and F.

20

Immunogenicity

Mice are immunized with glycopeptides and described herein, and *in vitro* restimulated CTL cells are tested for killing of target cells coated with different peptides and glycopeptides. Specificity P means that the CTL cells only recognize the peptide part G only th CHO part and GP only the combination.

25

BRIEF DESCRIPTION OF DRAWINGS

Figure 1

Chemical structure of compounds 1-15, described under the section
5 "Synthesis of spacer arm glycosides"

Figure 2

Chemical structure of glycopeptide products 21-25, described under the
section "Synthesis of glycopeptides".

Figure 3

CTL induced by pep9(NT60) more efficiently lysed both virus specific
and peptide-coated EL4 cells with 0.05 μ M pep 9(NT60) used for
restimulation than with 5 μ M and 0.5 μ M. CTL were generated by one
15 s.c. injection of 100 μ g pep 9(NT60). Immune spleen cells were
restimulated with syngeneic irradiated spleen cells in the presence of
pep 9(NT60) (A), 5 μ M; (B), 0.5 μ M and (C), 0.05 μ M for 5 days and
tested against EL4 untreated (O—O), NT60-infected (●—●), pep 9(NT60)-
coated (□—□).

Figure 4

Specificity of CTL induced by pep 9(PR8) (A) and pep 9(NT60) (B). The
effector CTL were tested against EL4 untreated (O—O), PR8-infected
(●—●), NT-60 infected (□—□), pep 9(PR8)-coated (■—■), pep 9(NT60)-
25 coated (Δ—Δ).

Figure 5

CTL responses primed and restimulated with live virus and peptides.
Mice were primed *in vivo* with PR8 live virus (A, B) or pep 9(PR8) (C, D),
30 and restimulated with PR8-infected spleen cells (A, C) or irradiated
spleen cells in the presence of 0.05 μ M pep 9(PR8) (B, D). Target cells
were EL4 untreated (O—O), PR8-infected (●—●), pep 9(PR8)-coated (□—□).

Figure 6

Kinetics of CTL activity induced by one s.c. injection of pep 9(PR8). Mice were primed with 100 µg pep 9(PR8) *in vivo* and restimulated *in vitro* with 5 µM pep 9(PR8) at day 2, 7, 10, 20 and 30 after priming. The generated CTL were tested against RMA untreated (O—O) and pep 9(PR8)-coated (●—●). Effector: target ratio, 60:1.

Figure 7

Peptide-induced cytotoxicity is mediated by CD8⁺ and H-2D^b restricted T cells. a: CTL were induced by pep 9(PR8). Equal numbers of CTL were then depleted for CD4⁺ and CD8⁺ T cells using the Dynabeads system. The remaining cells were then tested for lytic activity against pep 9(PR8)-coated EL4 cells. Untreated CTL (O—O), CD4⁺ depleted (●—●) and CD8⁺ depleted (□—□) were tested for lytic activity against pep 9(PR8)-coated EL4 cells. b: CTL induced by pep 9(PR8) were tested against PR8-infected EL4 (O—O), PR8-infected P815 (●—●) and PR8-infected L929 (□—□).

Figure 8

Peptide mediated H-2D^b up-regulation can be inhibited by chloroquine. RMA-S cells were incubated with influenza A (PR8) peptide NP366-374 (50 µM) in the presence or absence of chloroquine for 6 h. After incubation, cells were washed, stained with anti-D^b (28-14-8S) monoclonal antibody on ice for 30 minutes, washed and incubated with rabbit anti-mouse (F(ab')₂-FITC (F313, Dako, Copenhagen, Denmark) for 30 minutes. The stained cells were fixed with 1% formaldehyde and analysed in a FACScan flow cytometer (Becton Dickinson; Mountain View, CA).

Figure 9

Synthesis of glycopeptides (A and C). Glycopeptides were synthesised by coupling 2-bromoethyl 4-O-(α-D-galactopyranosyl)-β-D

galactopyranoside (compound 28) with peptides containing a thiol function. A cysteine moiety was added to the native peptide sequence at the aminotermius. Using caesium carbonate as a promoter, the nucleophilic property of the cysteine sulphur atom was utilised to create a thioether linkage between the peptide and the electrophilic spacer arm glycosidically linked to the carbohydrate moiety. The Gal₂ coupling reactions were performed in N,N-dimethylformamide. The coupling reactions were monitored by HPLC and were quenched by the addition of water containing 0.1% trifluoroacetic acid. The resulting glycopeptides (conjugates 29 (A) and 31 (C)) were purified by preparative HPLC and the molecular weights of the products were confirmed by FAB MS.

CTL assay. (B and D). Mice were immunized s.c. at the base of tail with 100 µg glycopeptide in incomplete Freund's Adjuvant. Ten days after priming, spleen cells were re-stimulated *in vitro* using 25×10^6 responder cells co-cultured with 25×10^6 irradiated (2500 rad) syngeneic spleen cells. Re-stimulation was done in the presence of glycopeptides at 0.05 µM in 50 ml tissue culture flasks with RPMI/10% FCS at 37°C for five days. RMA-S target cells were coated with 50 µM peptide for 1 hour at 37°C and washed. Cells were labelled with 100 µCi Na₂⁵¹CrO₄ for one hour at 37°C and washed twice. Effector lymphocytes were separated by Lymphoprep centrifugation and tested at different ratios with 5000 ⁵¹Cr-labelled target cells, using 150 µl/well in V-shaped microtiter wells. Microplates were centrifuged at 300 g and incubated for 4 hours at 37°C. After incubation plates were centrifuged again and 50 µl supernatants were assayed in a gamma scintillation counter.

Figure 10

Panel A: RMA-s cells were incubated for 24 hours at 26°C to induce expression of empty D^b MHC-1 chains at the cell surface, and at 37°C, and stained for D^b expression. Panel B: Removal of D^b expression by

pronase treatment of RMA-S cells, preincubated at 26°C. Pronase treatment for 2 h and 4 h. D^b removal is complete at 4 h.

Panels C and E: 26°C induced RMA-S cells were treated with the glycopeptides Gal₂-CSG11 (panel C) or Gal₂CAS10 (panel E), 300 µM for 2 h at 37°C, washed and stained for Gal₂ expression.

Panels D and F: Glycopeptide treated RMA-S cells as in panels C and E were pronase treated to remove Db and glycopeptide expression, washed and further incubated at 37°C to allow re-expression of glycopeptides and stained for Gal₂ expression.

Figure 11

Re-expression of D^b bound peptide on EL4 (A) and RMA-S (B) cells after pronase treatment as detected by specific CTL. EL4 (A) and RMA-S (B) cells were treated with peptide NP366-374 (100 µM) for 1.5 hours. After washing, cells were treated with Pronase E (ProE, 4 mg/ml in RPMI 1640) washed, and cultured at 37°C for 0, 1 and 3 hours and assayed in a 4 hour ⁵¹Cr release assay. CTL preparation has been previously described (Zhou et al., 1992 b). Briefly. C57B6/J female mice were immunized by one i.v. injection of 20 HAU of influenza A/PR/8/34 virus (a gift from Dr A. Douglas, National Institute for Medical Research, London) diluted in PBS. 1-4 weeks after the immunization, immune spleen cells were restimulated with irradiated, virus infected syngeneic spleen cells for 5 days.

Figure 12

The glycopeptide SGV12-Gal₂ (conjugate 30), used for generation of a carbohydrate-specific CTL response (cf. Example 4).

Figure 13

SGV12-Gal2 generated CTL cells were tested against EL-4 cells coated with peptides / glycopeptides as indicated in the panel. E/T, Effector:target ratio.

Figure 14(A) and (B)

RGY8-4h-Gal₂ generated CTL cells were tested against EL-4 cells coated with the indicated peptides/glycopeptides. E/T, Effector:target ratio.

REFERENCES

- Alchele *et al.* (1990), J. Exp. Med. 171, 1815-
- 5 Ansari, A.A., T. Frejd and G. Magnusson (1987), Carbohydr. Res. 161, 225-233.
- Brodin *et al.* (1988), Int. J. Cancer 42, 185-194.
- 10 Catelani, G. (1988), Carbohydr. Res. 182, 297-300.
- Crowle (1988), Infect. Immun. 56, 2769-2773.
- Dahmén *et al.* (1983 a), Carbohydrate Res. 113, 219-224.
- 15 Dahmén, J., T. Frejd, G. Grönberg, T. Lave, G. Magnusson and G. Noori (1983 b), Carbohydr. Res. 116, 303-307.
- Dahmén, J. *et al.* (1984), Carbohydr. Res. 127, 15-25.
- 20 Dasgupta, F. and P.J. Garegg (1988), Carbohydr. Res. 177, C13-C17.
- Domer *et al.* (1989), Infect. Immun. 57, 693-700.
- 25 Elofsson, M., Walse, B. and Kihlberg, J. (1991), Tetrahedron Lett. 32, 7613-7616.
- Falk *et al.* (1991), Nature 351, 290-296.
- 30 Fremont *et al.* (1992), Science 257, 919-927.
- Fung *et al.* (1990), Cancer Research 50, 4308-4314.
- Hansen *et al.* (1990), J. Virol. 64, 2833-2844
- 35 Hakomori (1991), Current Opinion in Immunology 3, 646-653.
- Harding (1992), Eur. J. Immunol. 22, 1865-1869.
- 40 Henningsson *et al.* (1987), Cancer Immunol. Immunother. 25, 231-241.
- Hochman *et al.* (1991), J. Immunol. 146, 1862-1867.
- Ishioka *et al.* (1992), J. Immunol. 148, 2446-2451.
- 45 Janeway (1991), Nature 353, 792.

- Jansson, K., S. Ahlfors, T. Frejd, J. Kihlberg and G. Magnusson (1988), J. Org. Chem. 53, 5629-5647.
- Jardetzky *et al.* (1991), Nature 353, 326-329.
- 5 Jondal & Pross (1975), Int. J. Cancer 15, 596-
- Jorgensen *et al.* (1992), Nature 355, 224-230.
- 10 Latron *et al.* (1992), Science 257, 964-967.
- Lönn, H. and K. Stenvall (1992), Tetrahedron Lett. 33, 115-116.
- 15 Magnusson, G., S. Ahlfors, J. Dahmén, K. Jansson, U. Nilsson, G. Noori, K. Stenvall and A. Tjörnebo (1982), J. Org. Chem. 55, 3932-3946.
- Madden *et al.* (1991), Nature 353, 321-325.
- Marra, A. and P. Sinaÿ (1990), Carbohydr. Res. 195, 303-308.
- 20 Marra, A. and P. Sinaÿ (1989), Carbohydr. Res. 187, 35-42.
- Masazuni *et al.* (1992), Science 257, 927-934.
- 25 Moll *et al.* (1989), Infect. Immun. 57, 3349-3356
- Morein *et al.* (1984), Nature 308, 457-460.
- 30 Murase, T., H. Ishida, M. Kiso and A. Hasegawa (1989), Carbohydr. Res. 188, 71-80.
- Nores *et al.* (1987), J. Immunol. 139, 3171-3176.
- 35 Oettgen, H.F., Ed. (1989). Gangliosides and Cancer. pub. VCH.
- Oldstone *et al.* (1988), J. Exp. Med. 168, 559-570.
- Or, Y.S., Clark, R.F. & Luly, J.R. (1991) J. Org. Chem. 56, 3146-3149.
- 40 Ortmann *et al.* (1992), J. Immunol. 148, 1445-1450.
- Otten *et al.* (1992), J. Immunol. 148, 3723-3732.
- Portukalain *et al.* (1979), Eur. J. Biochem. 94, 19-23.
- 45 Ray, A. *et al.* (1992), J. Am. Chem. Soc. 114, 2256-2257.
- Renaud, P. and D. Seebach (1986), Helv. Chim. Acta 69, 1704-1710.

- Robertsson *et al.* (1982), *Infect. Immun.* 37, 737-748
- Rötzschke *et al.* (1990), *Nature* 348, 252-254.
- 5 Rötzschke & Falk (1991), *Immunology Today* 12, 447-455.
- Sällberg *et al.* (1991), *Immunol. Lett.* 30, 59-
- Stauss (1991), *Current Biology* 1, 328-330.
- 10 Thurin, Hackett, Otvos & Loibner, *In Thurin: Abberant Glycosylation in Human Melanoma and Carcinoma* (Doctorate in medical science, University of Gothenburg, 1991, ISBN-91-628-0389-1).
- 15 Townsend *et al.* (1985), *Cell* 42, 457-
- Townsend *et al.* (1986), *Cell* 44, 959-
- Tsomides & Eisen (1991) *J. Biol. Chem.* 266, 3357-3360.
- 20 Van Bleek & Nathenson (1990), *Nature* 348, 213-216.
- Wiels *et al.* (1981) *Proc. Natl. Acad. Sci. USA* 78, 6485-
- 25 Yang, C.C., Marlowe, C.K. & Kania, R. (1991) *J. Am. Chem. Soc.* 113, 3177-3178.
- Zhou *et al.* (1992 a), *J. Immunol. Methods* 153, 193-200.
(Published in August 1992.)
- 30 Zhou *et al.* (1992 b), *Eur. J. Immunol.* 22, 3085-3090.
(Published in November 1992.)

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: AB ASTRA
- (B) STREET: Kvarnbergagatan 16
- (C) CITY: Sodertalje
- (E) COUNTRY: Sweden
- (F) POSTAL CODE (ZIP): S-151 85
- (G) TELEPHONE: +46-8-55 32 60 00
- (H) TELEFAX: +46-8-55 32 88 20
- (I) TELEX: 19237 astra s

(ii) TITLE OF INVENTION: New Active Compounds

(iii) NUMBER OF SEQUENCES: 27

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(vi) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: SE 9201338-2
- (B) FILING DATE: 28-APR-1992

(vi) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: SE 9202553-5
- (B) FILING DATE: 07-SEP-1992

(vi) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: SE 9203897-5
- (B) FILING DATE: 23-DEC-1992

(vi) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: SE 9301141-9
- (B) FILING DATE: 06-APR-1993

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Ala Ser Asn Glu Asn Met Glu Thr Met
1 5

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Ser Gly Pro Ser Asn Thr Pro Pro Glu Ile
1 5 10

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Gly Ile Leu Gly Phe Val Phe Thr Leu
1 5

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Lys Ile Ala Ser Asn Glu Asn Met Asp Ala Met Glu Ser Ser Thr Leu
1 5 10 15

Glu Cys

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Lys Ile Ala Ser Asn Glu Asn Met Glu Thr Met Glu Cys
1 5 10

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Lys Ala Ser Asn Glu Asn Met Glu Thr Met Cys
1 5 10

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

Cys Ala Ser Asn Glu Asn Met Glu Thr Met
1 5 10

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

Cys Ser Gly Pro Ser Asn Thr Pro Pro Glu Ile
1 5 10

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

Ser Gly Val Glu Asn Pro Gly Gly Tyr Cys Leu Thr
1 5 10

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

Lys Gln Ile Ala Ser Asn Glu Asn Met Glu Thr Met Glu Ser Cys
1 5 10 15

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 11 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

Lys Gly Pro Ser Asn Thr Pro Pro Glu Ile Cys
1 5 10

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 13 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

Lys Ser Gly Pro Ser Asn Thr Pro Pro Glu Ile His Cys
1 5 10

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 9 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

Cys Arg Gly Tyr Val Tyr Gln Gly Leu
1 5

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 9 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

Cys Ala Pro Gly Asn Tyr Pro Ala Leu
1 5

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

Met Val Val Lys Leu Gly Glu Phe Tyr Asn Gln Met Met
1 5 10

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

Cys Pro Thr Asn Gln Gln Val Val Leu Glu Gly Thr Asn Lys Thr Asp
1 5 10 15

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

Met Gln Ile Arg Gly Phe Val Tyr Phe Val Glu Thr
1 5 10

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

Leu Ser Pro Gly Met Met Met Gly Met Phe Asn Met
1 5 10

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

Asp Tyr Gly Ile Leu Gln Ile Asn Ser Arg
1 5 10

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

Phe Ala Pro Gly Asn Tyr Pro Ala Leu
1 5

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

(A) NAME/KEY: Modified-site
(B) LOCATION: 6..7
(D) OTHER INFORMATION: /label= Xaa
/note= "homocysteine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

Ala Ser Asn Glu Asn Xaa Glu Thr Met
1 5

(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

- (ix) FEATURE:
 (A) NAME/KEY: Modified-site
 (B) LOCATION: 4..5
 (D) OTHER INFORMATION: /label= Xaa
 /note= "homocysteine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:
Ala Ser Asn Xaa Asn Met Glu Thr Met
1 5

(2) INFORMATION FOR SEQ ID NO: 23:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 10 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

- (ix) FEATURE:
 (A) NAME/KEY: Modified-site
 (B) LOCATION: 6..7
 (D) OTHER INFORMATION: /label= Xaa
 /note= "homocysteine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:
Ser Gly Pro Ser Asn Xaa Pro Pro Glu Ile
1 5 10

(2) INFORMATION FOR SEQ ID NO: 24:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 10 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

- (ix) FEATURE:
 (A) NAME/KEY: Modified-site
 (B) LOCATION: 4..5
 (D) OTHER INFORMATION: /label= Xaa
 /note= "homocysteine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:
Ser Gly Pro Xaa Asn Thr Pro Pro Glu Ile
1 5 10

(2) INFORMATION FOR SEQ ID NO: 25:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 8 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

- (ix) FEATURE:
 (A) NAME/KEY: Modified-site
 (B) LOCATION: 4..5
 (D) OTHER INFORMATION: /label= Xaa
 /note= "homocysteine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

Arg Gly Tyr Xaa Tyr Gln Gly Leu
1 5

(2) INFORMATION FOR SEQ ID NO: 26:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 9 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

- (ix) FEATURE:
 (A) NAME/KEY: Modified-site
 (B) LOCATION: 5..6
 (D) OTHER INFORMATION: /label= Xaa
 /note= "homocysteine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

Phe Ala Pro Gly Xaa Tyr Pro Ala Leu
1 5

(2) INFORMATION FOR SEQ ID NO: 27:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 8 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

Arg Gly Tyr Val Tyr Gln Gly Leu
1 5

The claims defining the invention are as follows:

1. A peptide/carbohydrate conjugate capable of generating T cell immunity against a carbohydrate structure, said conjugate comprising (i) a peptide component capable of binding a MHC class I molecule; and (ii) a carbohydrate component having the immunogenic specificity of said carbohydrate structure.
2. A conjugate according to claim 1 generating T cell immunity against a tumor-associated carbohydrate structure, said conjugate comprising (i) a peptide component capable of binding a MHC class I molecule; and (ii) a carbohydrate component having the immunogenic specificity of said tumor-associated carbohydrate structure.
3. A conjugate according to claim 1 generating T cell immunity against a carbohydrate structure expressed on infectious agents and/or infected host cells, said conjugate comprising (i) a peptide component capable of binding a MHC class I molecule; and (ii) a carbohydrate component having the immunogenic specificity of said carbohydrate structure expressed on infectious agents and/or infected host cells.
4. A conjugate according to any one of claims 1-3 wherein the peptide component consists of 5-25 amino acids.
5. A conjugate according to any one of claims 1-4 wherein the peptide component consists of 8-12 amino acids.
6. A conjugate according to any one of claims 1-5 wherein the peptide component consists of 9 amino acids.



7. A conjugate according to any one of claims 1-6 wherein the peptide component is capable of binding a human MHC class I molecule.
8. A conjugate according to any one of claims 1-7 wherein the peptide component has the amino acid V, I, L, or T in position 6.
9. A conjugate according to claim 8 wherein the peptide component has the amino acid V in position 6.
10. A conjugate according to any one of claims 1-9 wherein the peptide component has the amino acid V or L in C-terminal position.
11. A conjugate according to any one of claims 1-10 wherein the peptide component has the amino acid L in C-terminal position.
12. A conjugate according to any one of claims 1-11 wherein the peptide component has the sequence G I L G F V F T L (SEQ ID NO: 3 in the Sequence Listing).
13. A conjugate according to any one of claims 1-12 wherein the carbohydrate component is conjugated to the peptide component in a position to bind a hypervariable region of the T cell receptor.
14. A conjugate according to any one of claims 1-13 wherein the carbohydrate component is sized to enable the T cell receptor to encompass an epitope of said carbohydrate structure.
15. A conjugate according to any one of claims 1-14 wherein the carbohydrate component is any of those defined in Table 1 of the specification.



16. A pharmaceutical formulation containing a conjugate according to any one of claims 1-15 as active ingredient.
17. A conjugate according to any one of claims 1-15 for use in therapy.
18. A method of stimulating the generation of cytotoxic T cells (CTLs) in a patient, wherein said CTLs have the potential to destroy or attenuate cells presenting a characteristic disease-associated carbohydrate structure, which comprises administering to the patient an effective dose of a conjugate as claimed in any one of claims 1-15, or of a formulation as claimed in claim 16.
19. A method of generating cytotoxic T cells (CTLs) which have the potential to destroy or attenuate disease-associated cells presenting a characteristic disease-associated carbohydrate structure, which comprises contacting a cell population with a conjugate as claimed in any one of claims 1-15, or with a formulation as claimed in claim 16, wherein said cell population includes (a) cells possessing MHC class I molecules capable of binding to the peptide component of said conjugate and (b) cells capable of being converted to CTLs having the said potential on interaction with cells (a) having said conjugate bound to a MHC class I molecule.
20. A method according to claim 19 wherein said cell population comprises cells which have been removed or isolated from an animal body.
21. A method according to claim 20 which includes the step of introducing so-produced CTLs into the body of an animal possessing said disease-associated cells.
22. The use of a conjugate according to any one of claims 1-15 in the manufacture of a pharmaceutical composition for stimulating the production of cytotoxic T cells (CTLs) in a patient, wherein said



CTLs have the potential to destroy or attenuate cells presenting a characteristic disease-associated carbohydrate structure.

23. The use of a conjugate according to any one of claims 1-15 in the manufacture of a pharmaceutical composition for producing cytotoxic T cells (CTLs) which have the potential to destroy or attenuate disease-associated cells presenting a characteristic disease-associated carbohydrate structure by a procedure which comprises contacting a cell population with said conjugate, wherein said cell population includes (a) cells possessing MHC class I molecules capable of binding to the peptide component of said conjugate and (b) cells capable of being converted to CTLs having the said potential on interaction with cells (a) having said conjugate bound to a MHC class I molecule.

24. The use according to claim 23 wherein said cell population comprises cells which have been removed or isolated from an animal body.

25. The use according to claim 24 which includes the step of introducing so-produced CTLs into the body of an animal possessing said disease-associated cells.

26. The use of a conjugate as claimed in any one of claims 1-15 in the manufacture of a pharmaceutical composition for inducing a desired immunological state in a patient, wherein said immunological state results from an interaction between said conjugate and an MHC class I molecule whereby a cellular component of the immune system is stimulated to induce a response specifically associated with said carbohydrate structure.



27. A method for inducing a desired immunological state in a patient, wherein said immunological state results from an interaction between a peptide/carbohydrate conjugate and an MHC class I molecule whereby a cellular component of the immune system is stimulated to induce a response specifically associated with a carbohydrate structure, which comprises administering an effective dose of a conjugate as claimed in any one of claims 1-15, or of a formulation as claimed in claim 16.

28. The use of a conjugate according to any one of claims 1-15 in the manufacture of a medicament for the treatment of malignant diseases.

29. The use according to claim 28 in the manufacture of a medicament for the treatment of melanoma, breast cancer, lung cancer or gastrointestinal cancer.

30. The use of a conjugate according to any one of claims 1-15 in the manufacture of a medicament for the treatment of infectious diseases.

31. The use according to claim 30 in the manufacture of a medicament for the treatment of infectious diseases caused by parasitic agents.

32. The use according to claim 31 in the manufacture of a medicament for the treatment of infectious diseases caused by intracellular pathogens.

33. A method for the treatment of malignant diseases by administering to a host in need of such treatment of a therapeutically effective amount of a conjugate according to any one of claims 1-15, or of a formulation as claimed in claim 16.



34. A method according to claim 33 for the treatment of melanoma, breast cancer, lung cancer or gastrointestinal cancer.
- 5 35. A method for the treatment of infectious diseases by administering to a host in need of such treatment of a therapeutically effective amount of a conjugate according to any one of claims 1-15, or of a formulation as claimed in claim 16.
36. A method according to claim 35 for the treatment of infectious diseases caused by parasital agents.
- 10 37. A method according to claim 35 for the treatment of infectious diseases caused by intracellular agents.
- 15 38. A process for producing a peptide/carbohydrate conjugate as claimed in any one of claims 1 to 15 which comprises one or more of the following procedure steps:
- (a) synthesizing the peptide component of the conjugate by a known technique of peptide synthesis,
 - 20 (b) synthesizing the carbohydrate component of the conjugate by a known technique of carbohydrate synthesis,
 - (c) protecting one or more -COOH, OH, -NH₂ or -SH groups of the peptide component prior to coupling the peptide component covalently to the carbohydrate component,
 - 25 (d) protecting one or more -OH, -COOH, -NH₂, -CHO or =CO groups of the carbohydrate component prior to coupling the carbohydrate component covalently to the peptide component,
 - (e) activating an unprotected -OH, -COOH, -NH₂, -CHO, or =CO group of the carbohydrate component,
 - (f) activating an unprotected -OH, -COOH, -NH₂, or -SH group of the peptide component.



5

- (g) reacting at least one of the carbohydrate component and the peptide component with a bifunctional linking reagent,
- (h) reacting the carbohydrate component and the peptide component covalently so as to form the desired conjugate, said components being suitably protected, activated and/or reacted with a bifunctional linking reagent,
- (i) subjecting an intermediated protected peptide/carbohydrate conjugate to a deprotecting procedure.

DATED this 29th day of July 1996

ASTRA AKTIEBOLAG,
By its Patent Attorneys,
E. F. WELLINGTON & CO.,
By:

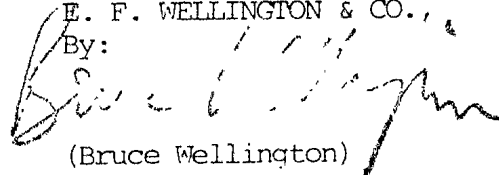
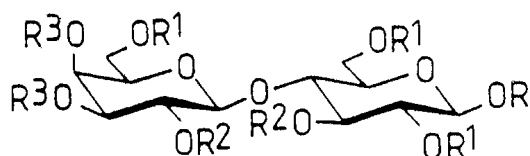
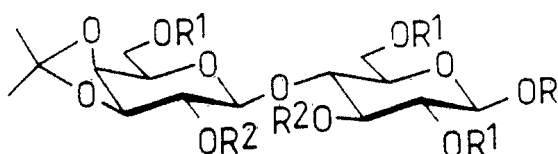

(Bruce Wellington)



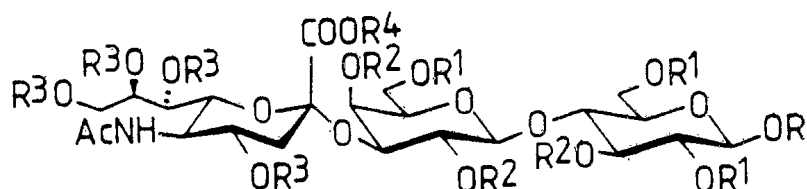
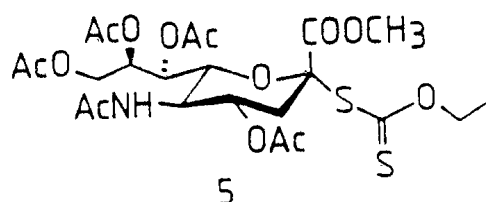
Fig. 1.



- 1: $R = \text{CH}_2\text{CH}_2\text{Si}(\text{CH}_3)_3$; $R^1, R^2, R^3 = \text{Ac}$
 2: $R = \text{CH}_2\text{CH}_2\text{Si}(\text{CH}_3)_3$; $R^1, R^2, R^3 = \text{H}$
 4: $R = \text{CH}_2\text{CH}_2\text{Si}(\text{CH}_3)_3$; $R^1, R^2 = \text{Bzl}$; $R^3 = \text{H}$
 10: $R = \text{CH}_2\text{CH}_2\text{Br}$; $R^1, R^2, R^3 = \text{Ac}$
 11: $R = \text{CH}_2\text{CH}_2\text{Br}$; $R^1, R^2, R^3 = \text{H}$
 13: $R = \text{CH}_2\text{CH}_2\text{Br}$; $R^1 = \text{Bz}$; $R^2, R^3 = \text{H}$



- 3: $R = \text{CH}_2\text{CH}_2\text{Si}(\text{CH}_3)_3$; $R^1, R^2 = \text{Bzl}$
 12: $R = \text{CH}_2\text{CH}_2\text{Br}$; $R^1, R^2 = \text{H}$



- 6: $R = \text{CH}_2\text{CH}_2\text{Si}(\text{CH}_3)_3$; $R^1, R^2, R^3 = \text{Ac}$; $R^4 = \text{CH}_3$
 7: $R, R^1, R^2, R^3 = \text{Ac}$; $R^4 = \text{CH}_3$
 8: $R = \text{CH}_2\text{CH}(\text{CH}_2\text{Br})_2$; $R^1, R^2, R^3 = \text{Ac}$; $R^4 = \text{CH}_3$
 9: $R = \text{CH}_2\text{CH}(\text{CH}_2\text{Br})_2$; $R^1, R^2, R^3 = \text{H}$; $R^4 = \text{Na}^+$
 14: $R = \text{CH}_2\text{CH}_2\text{Br}$; $R^1 = \text{Bz}$; $R^2 = \text{H}$; $R^3 = \text{Ac}$; $R^4 = \text{CH}_3$
 15: $R = \text{CH}_2\text{CH}_2\text{Br}$; $R^1, R^2, R^3 = \text{H}$; $R^4 = \text{Na}^+$

Fig. 2.

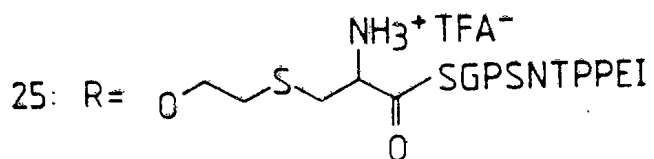
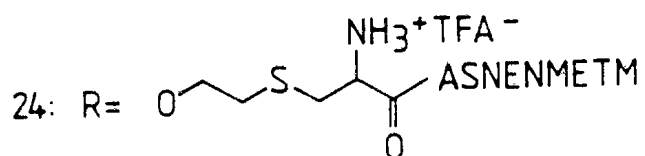
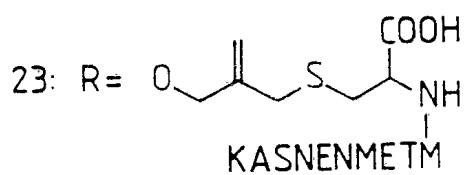
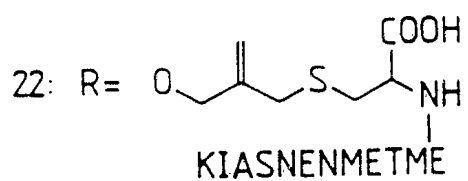
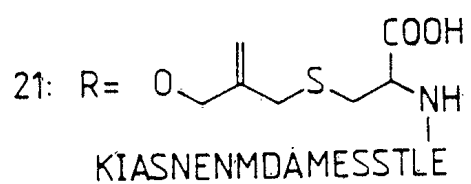
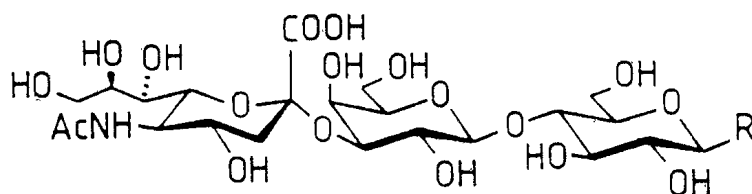


Fig.3.

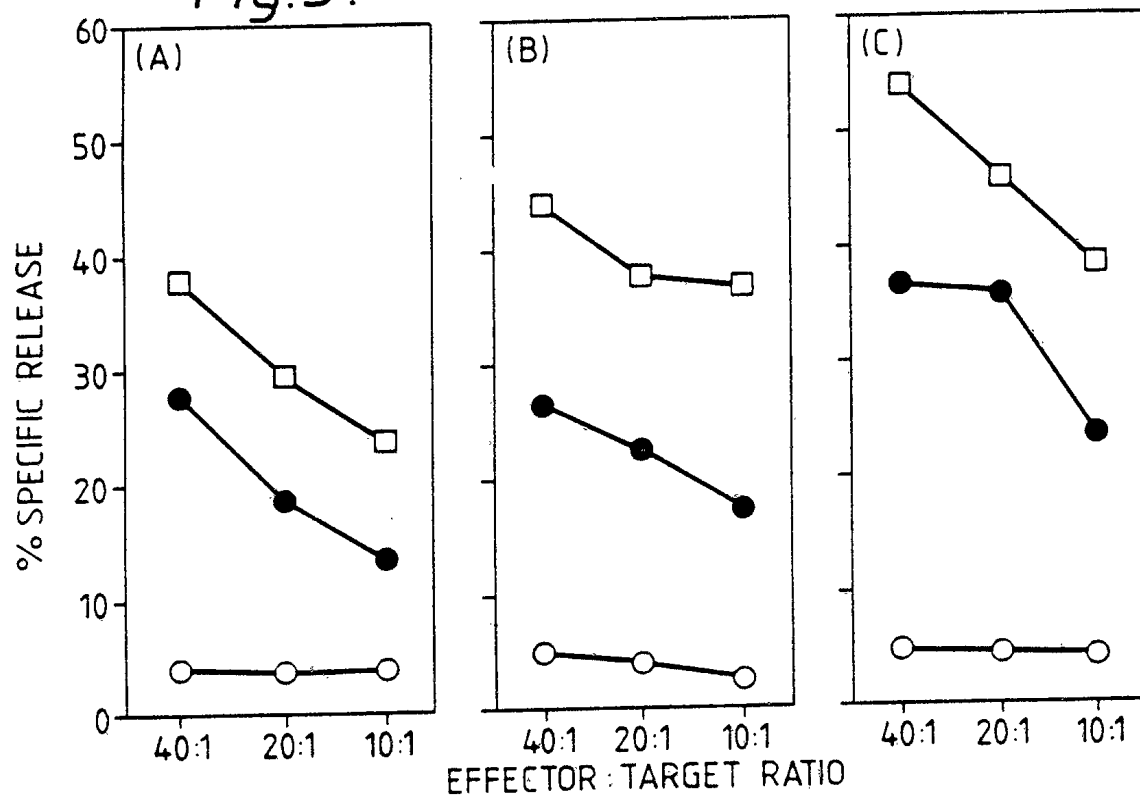
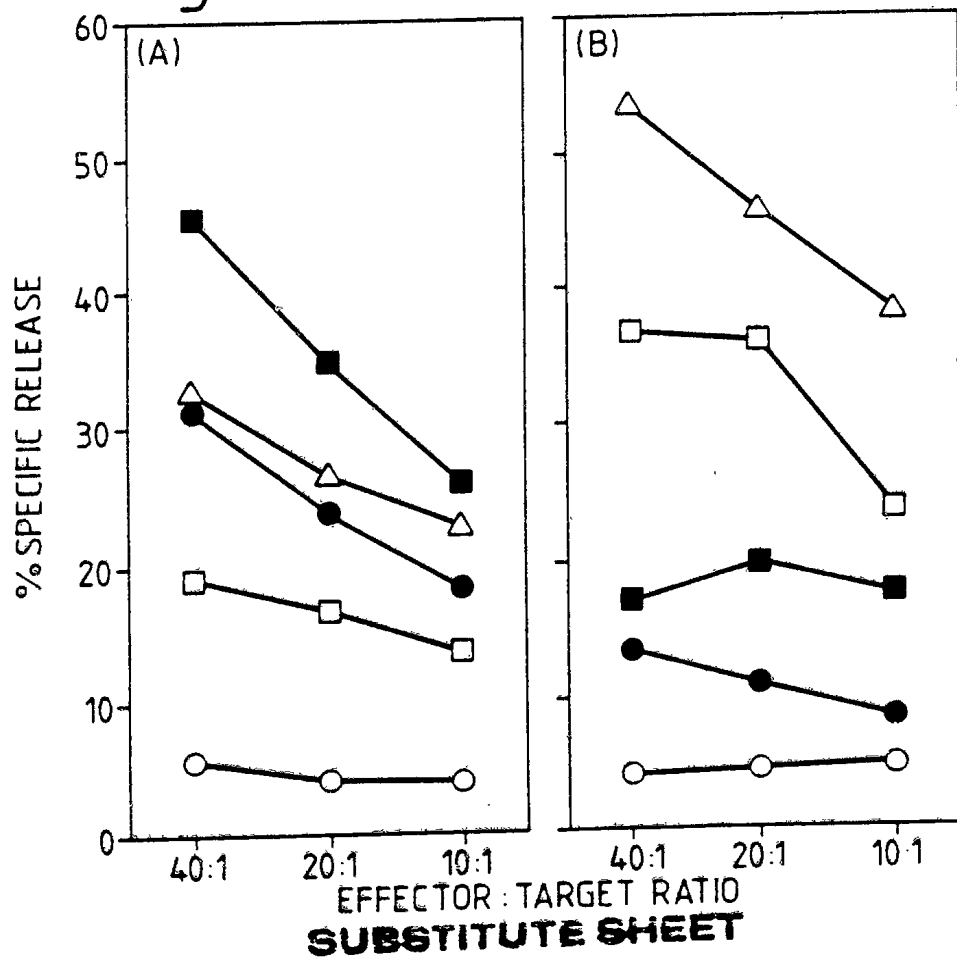


Fig.4.



SUBSTITUTE SHEET

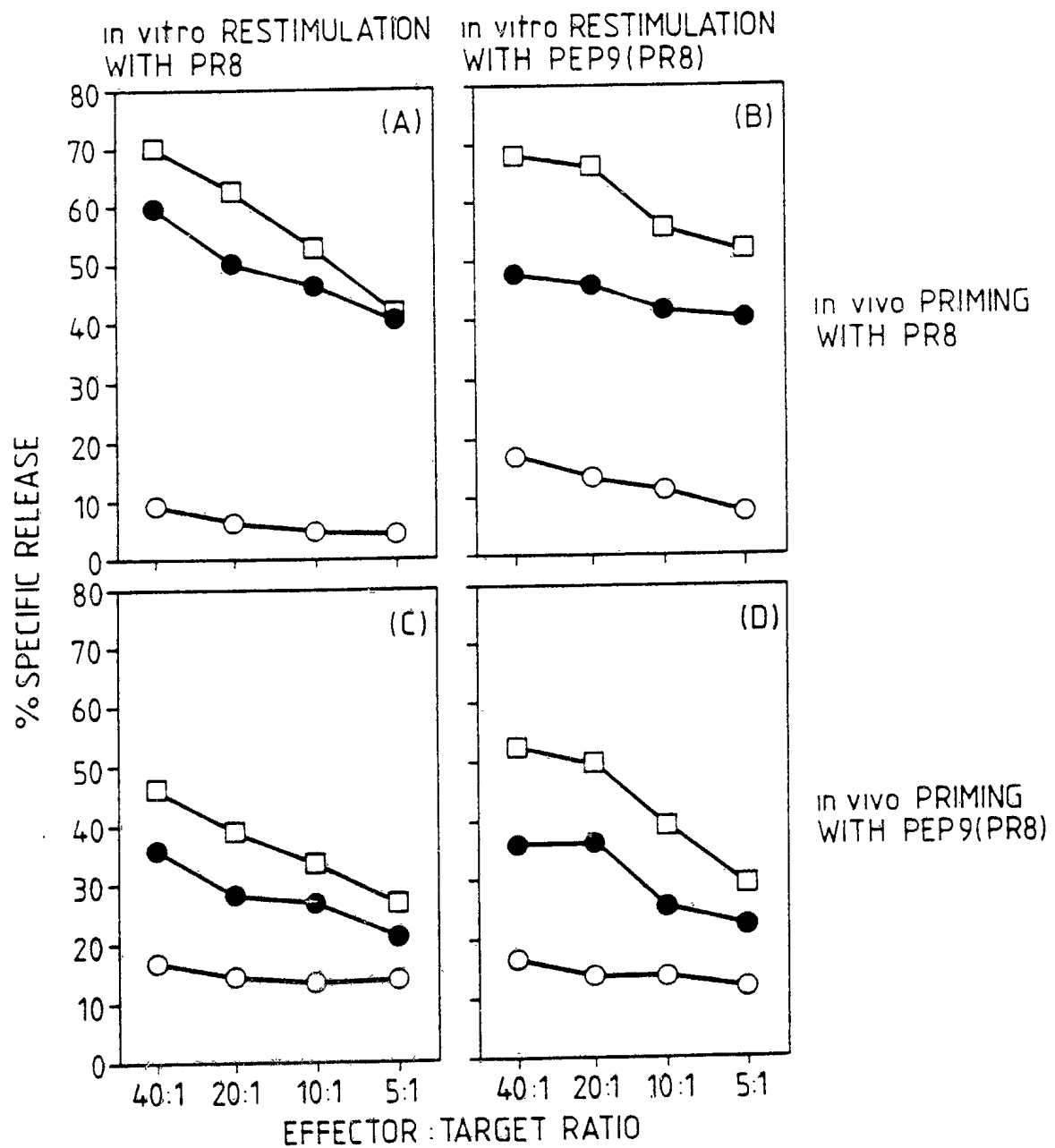
Fig. 5.

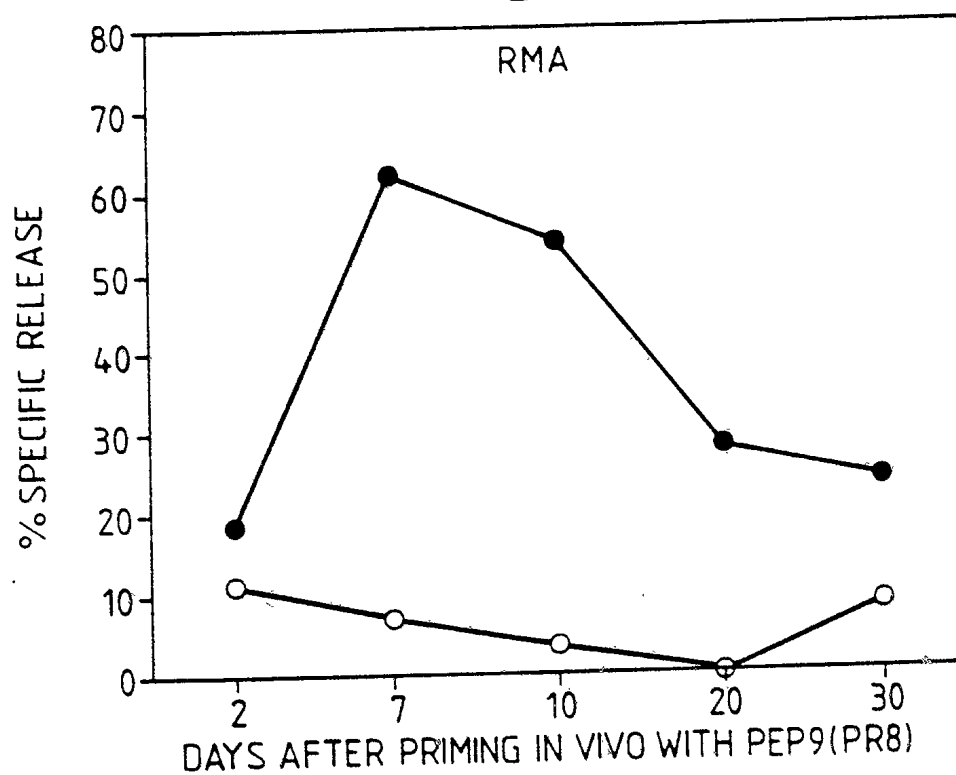
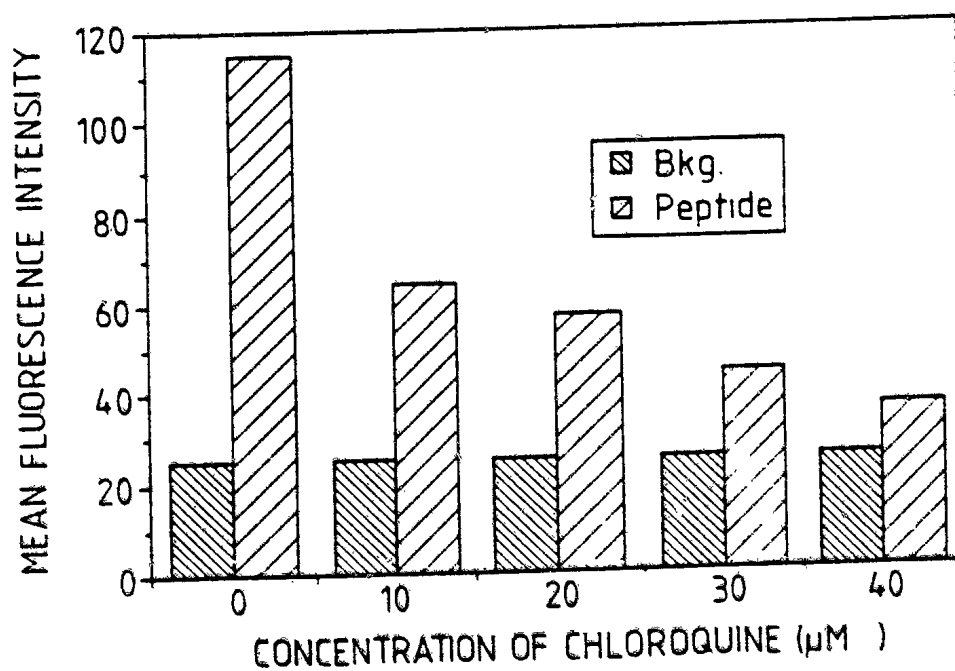
Fig. 6.*Fig. 8.*

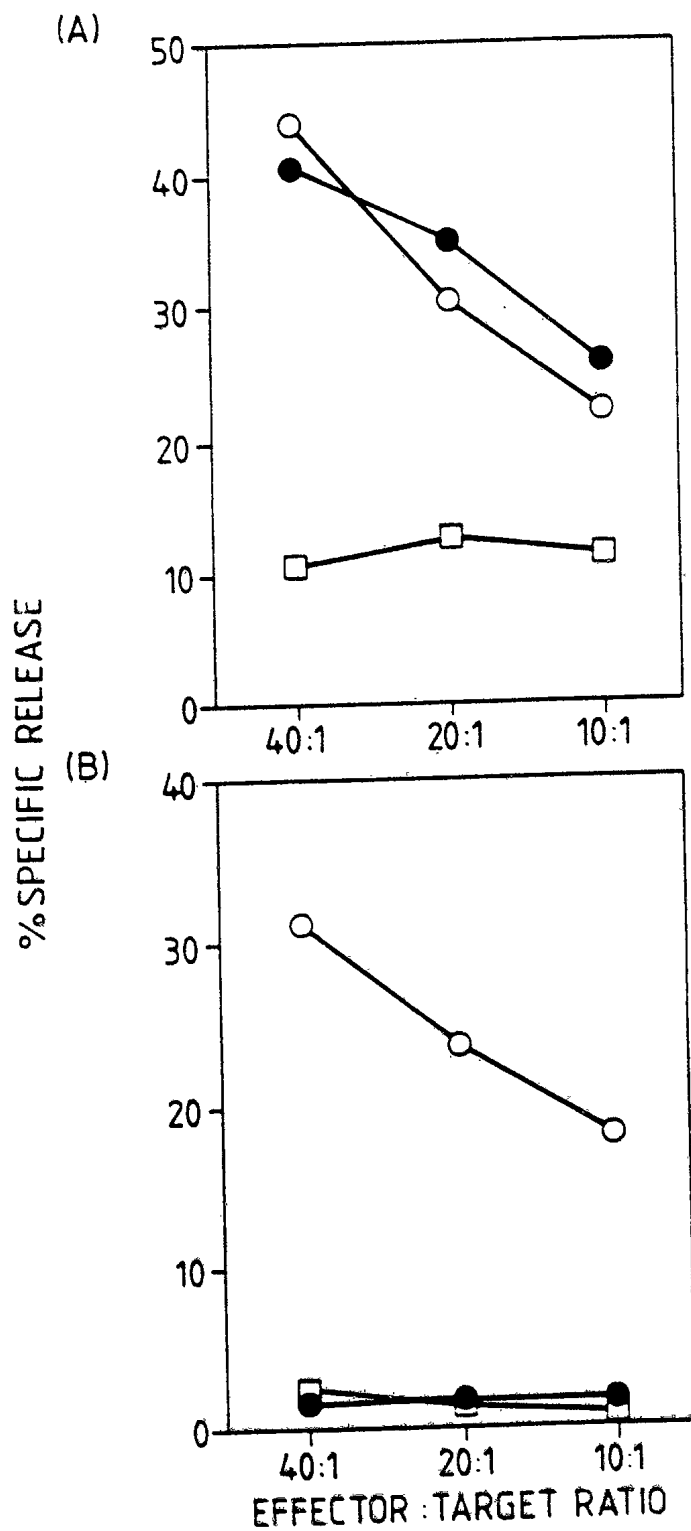
Fig. 7.

Fig. 9.

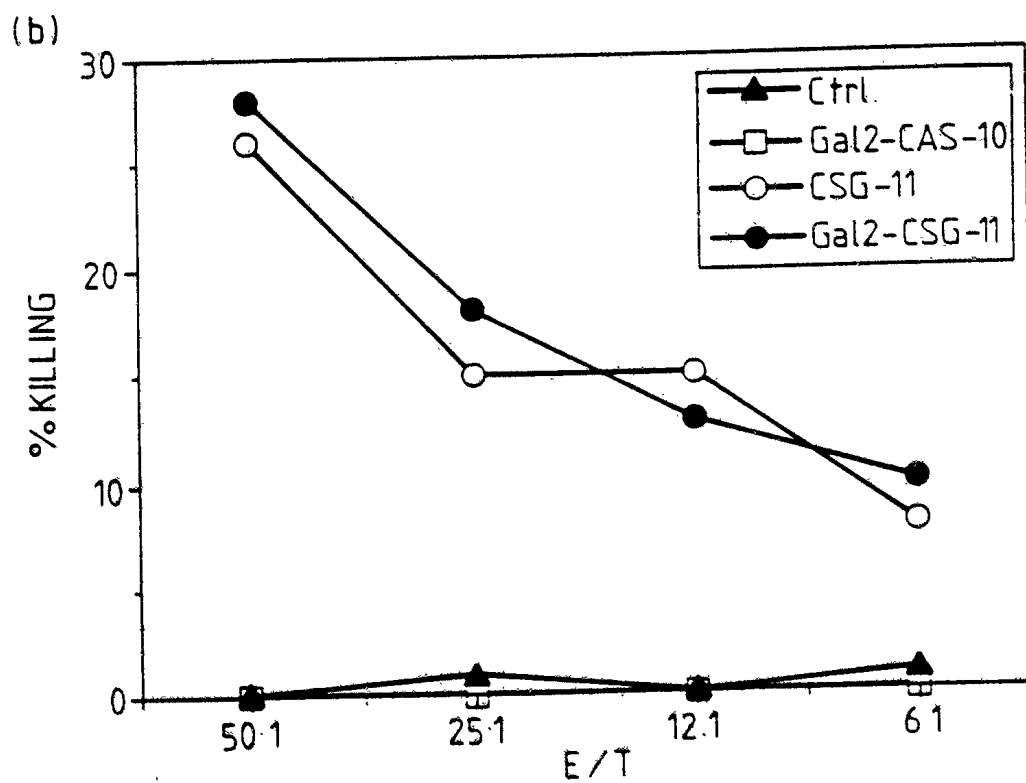
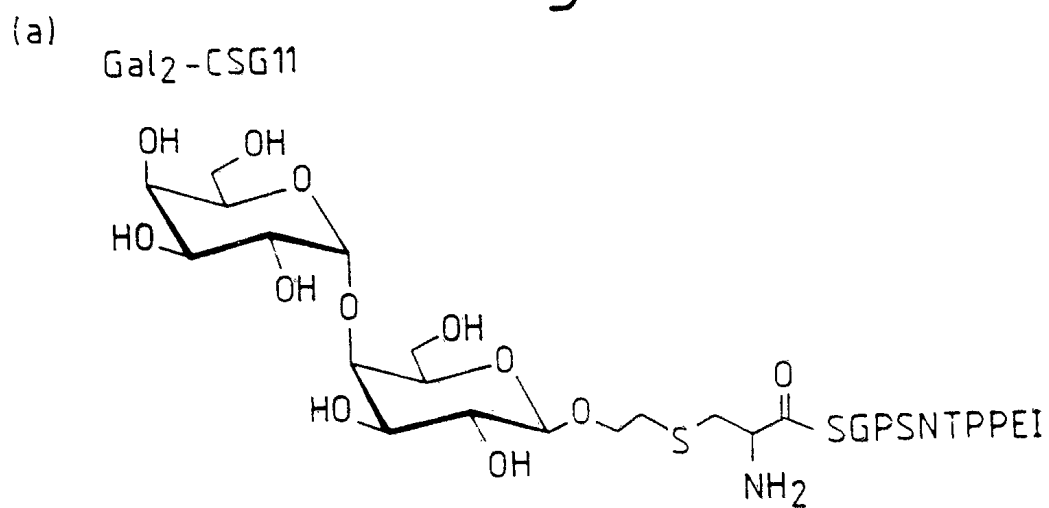
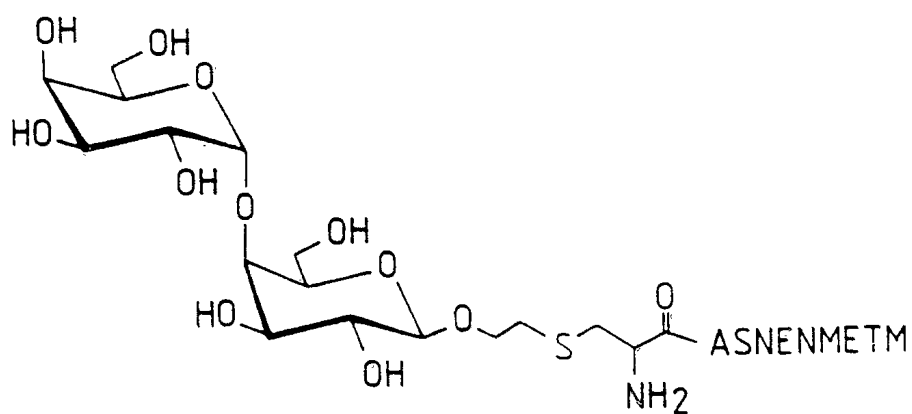


Fig. 9(Cont).

(c)

Gal₂-CAS10

(d)

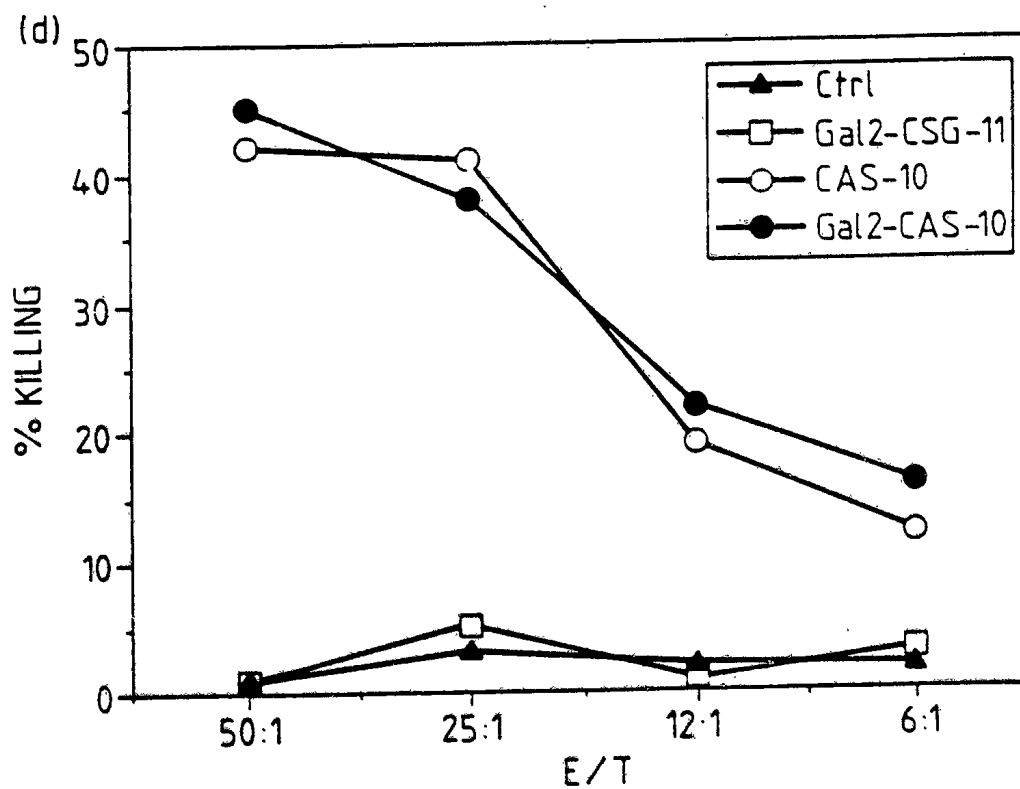


Fig.10.

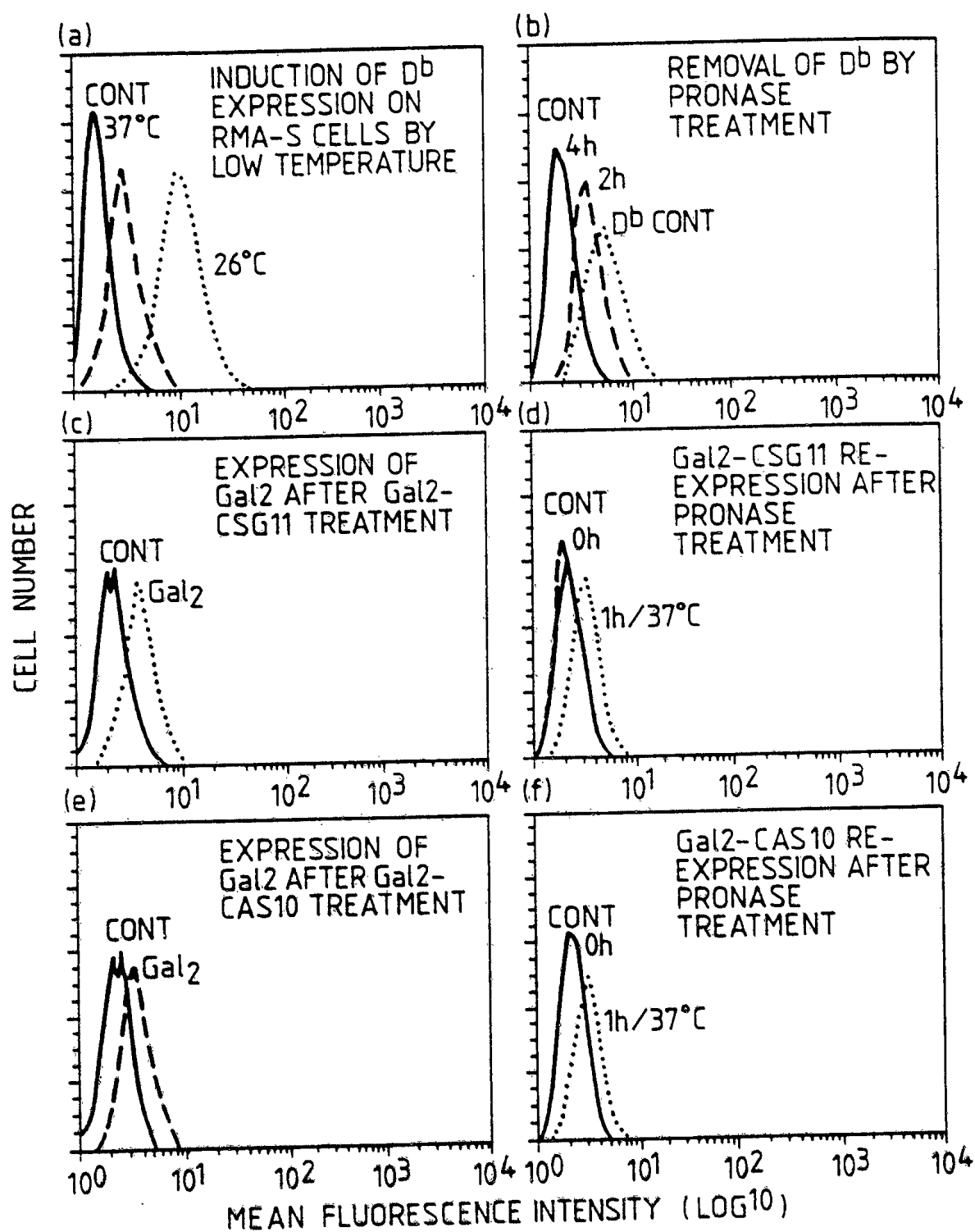


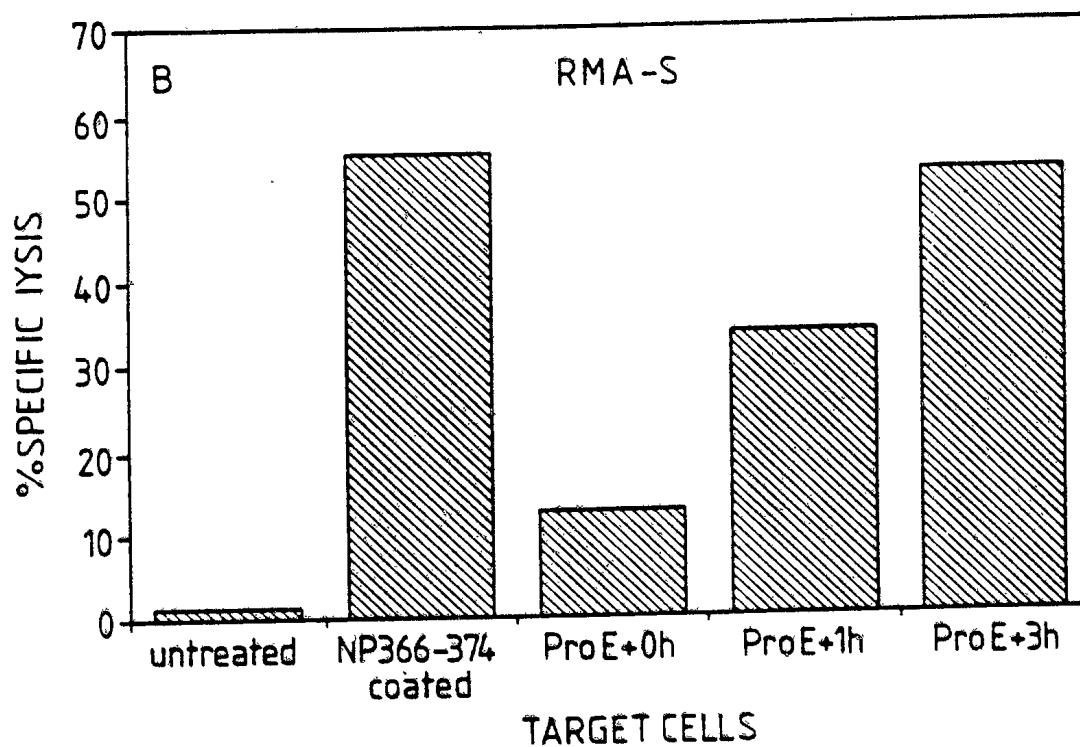
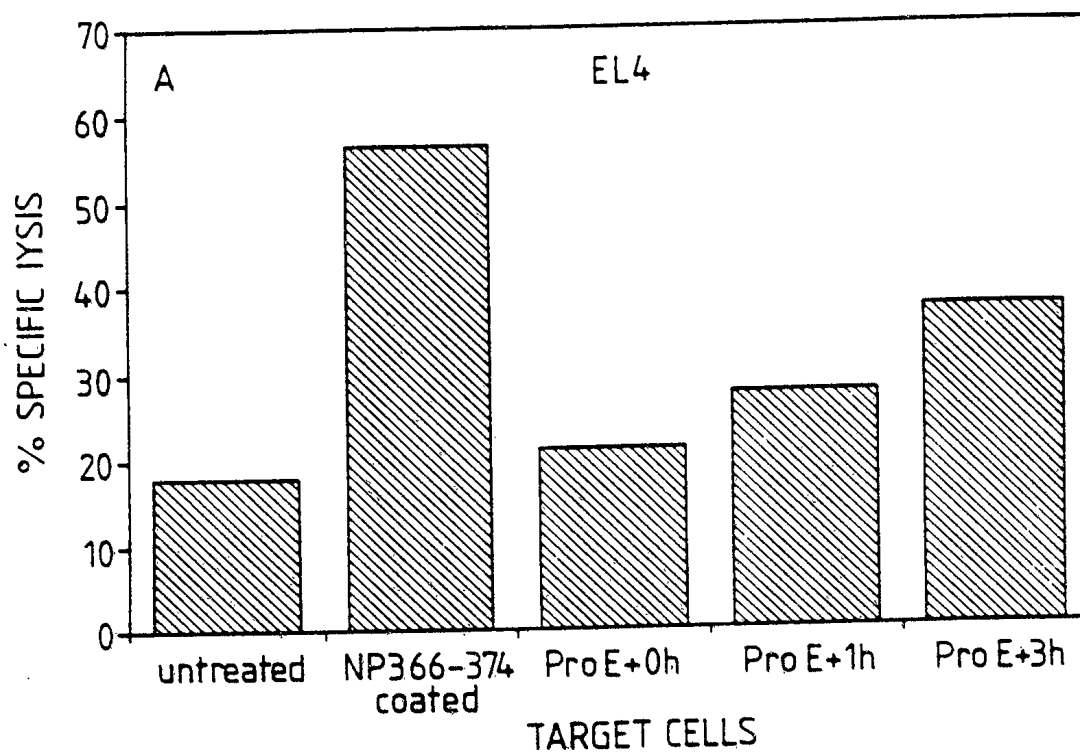
Fig.11.

Fig.12.

Gal-Gal-SGV-12

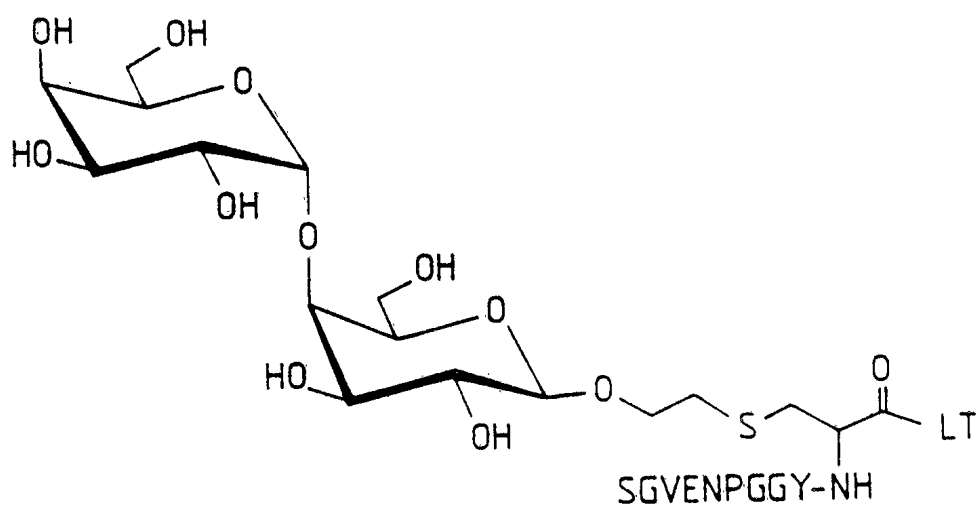


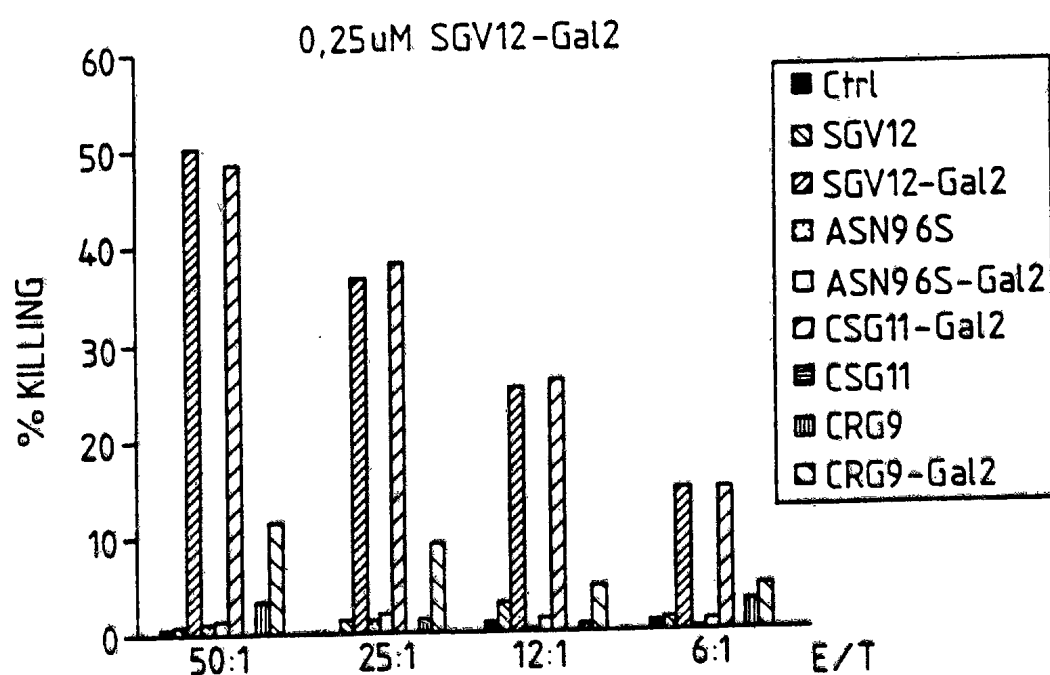
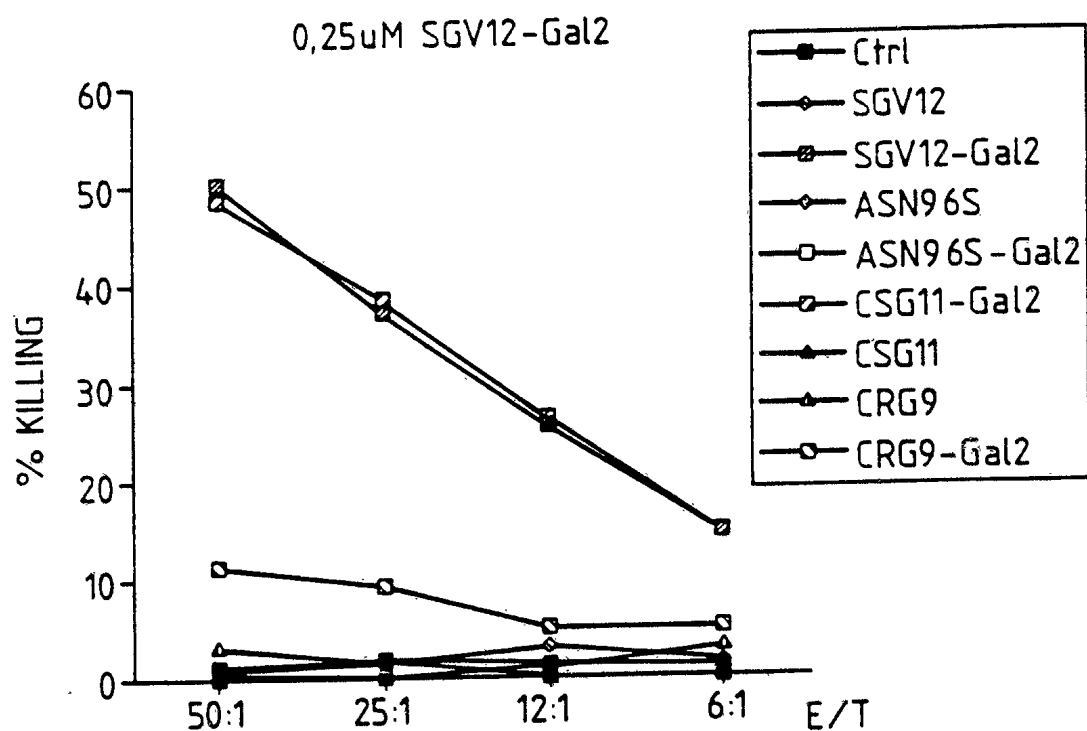
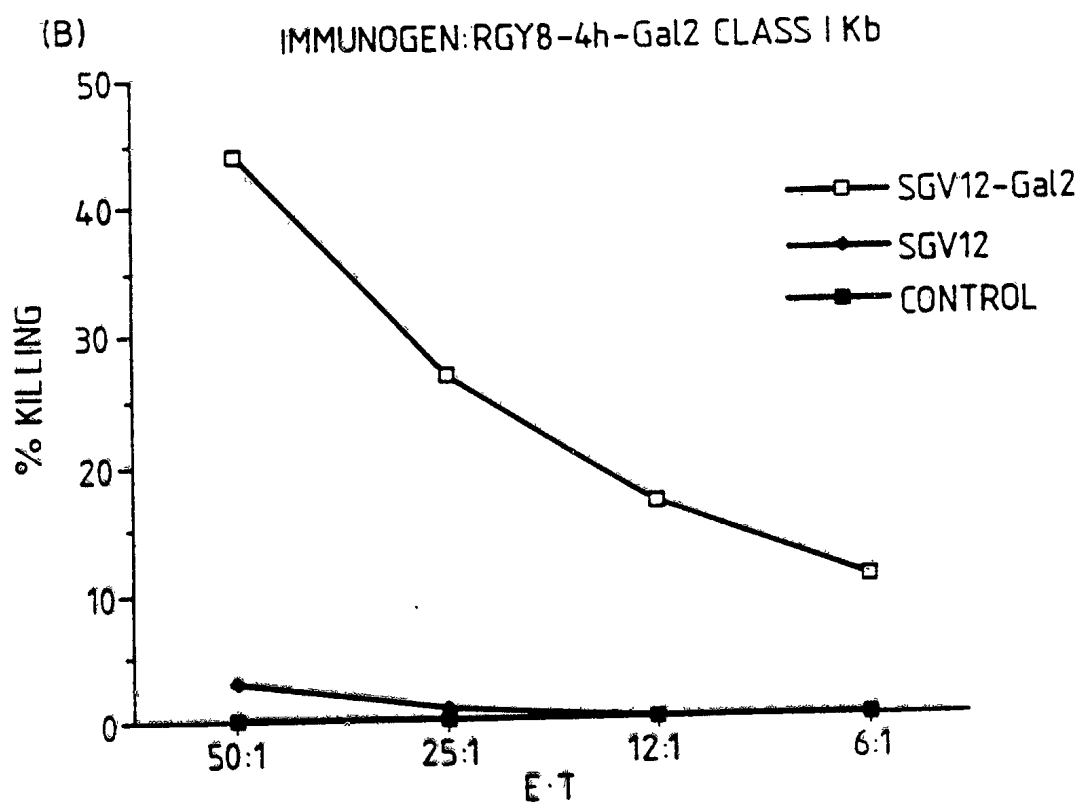
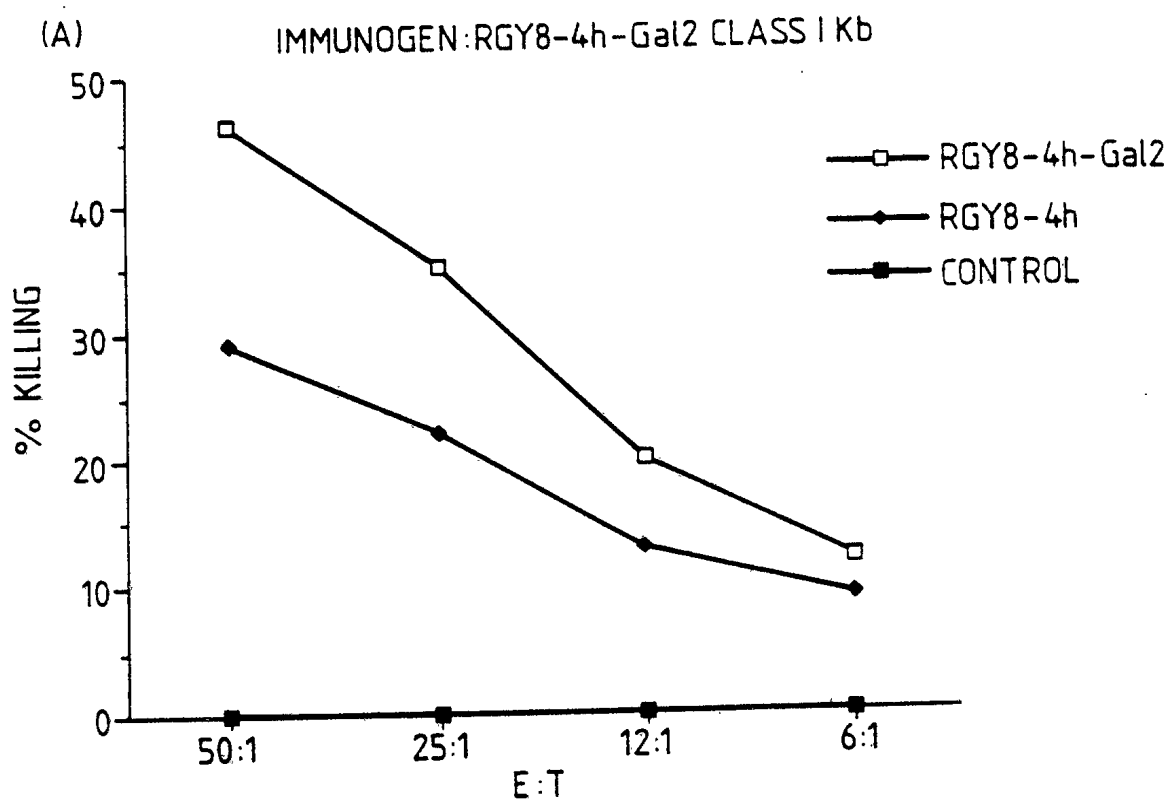
Fig.13.

Fig.14.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 93/00353

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

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

1. ☒ Claims Nos.: 18, 21, 25, 33-37
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although these claims are directed to a method of treatment of, and the diagnostic method practised on, the human body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 93/00353

A. CLASSIFICATION OF SUBJECT MATTER

IPC5: A61K 39/00, A61K 37/00, C07K 9/00

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC5: A61K, C07K

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

SE,DK,FI,NO classes as above

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

MEDLINE, CA, WPI

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	IMMUNOLOGY TODAY, Volume 12, No 12, 1991, Olaf Röttschke et al, "Naturally-occurring peptide antigens derived from the MHC class-I-restricted processing pathway", page 447 - page 455, see pages 450 and 454 --	1-17, 19, 20, 22-24, 26-32
Y	SCIENCE, Volume 257, August 1992, Masazumi Matsumura et al, "Emerging Principles for the Recognition of Peptide Antigens by MHC Class I Molecules" page 927 - page 934 --	1-17, 19, 20, 22-24, 26-32
A	WO, A1, 8907448 (REGENTS OF THE UNIVERSITY OF CALIFORNIA), 24 August 1989 (24.08.89), page 9, line 27 - page 11, line 33 --	1-3

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "B" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

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

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

24 August 1993

Name and mailing address of the ISA/
Swedish Patent Office
Box 5055, S-102 42 STOCKHOLM
Facsimile No. +46 8 666 02 86

Date of mailing of the international search report

125 -08- 1993

Authorized officer

Carl Olof Gustafsson
Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 93/00353

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CANCER IMMUNOL IMMUNOTHER, Volume 25, 1987, Carina M. Henningsson et al, "T cell recognition of a tumor-associated glycoprotein and its synthetic carbohydrate epitopes: stimulation of anticancer T cell immunity in vivo" page 231 - page 241 --	1-17,19,20, 22-24,26-32
Y	TETRAHEDRON LETTERS, Volume 31, No 41, 1990, Laszlo Otvos et al, "Automated solid-phase synthesis of glycopeptides. Incorporation of unprotected mono- and disaccharide units of N-glycoprotein antennae into cell epitopic peptides", page 5889 - page 5892, see page 5890, second paragraph and last paragraph --	1-17,19,20, 22-24,26-32
X	--	1,3-5,7,8, 13,14,38
A	National Library of Medline, NLM, File Medline, accession no. 87089861, Koprowski H: "Stimulation of cytotoxic T-lymphocyte responses by rabies virus glycoprotein and identification of an immunodominant domain", Mol Immunol 1986 Jul;23 (7): 733-41 --	1,3-5,7,8, 13,14
A	Dialog Information Services, File 154, Medline, Dialog accession no. 07932964, Medline accession no. 92070964, O'Neill HC: "Monoclonal antibodies which identify carbohydrate-defined MHC class I epitopes", Immunol Cell Biol, Jun 1991, 69 (Pt 3) p 159-65 --	1
A	National Library of Medicine, NLM, File Medline, accession no. 86060979, Pimlott NJ: "Glycopeptides inhibit allospecific cytotoxic T lymphocyte recog- nition in an MHC-sepcific manner", J Immunol 1986 Jan 136 (1) 6-11 --	1,2

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 93/00353

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	National Library of Medline, NLM, File Medline, accession no. 92218842, Ishioka GY: "MHC interaction and T cell recognition of carbohydrates and glycopeptides", J Immunol 1992 Apr 15; 148(8): 2446-51 --	1
A	JOURNAL OF IMMUNOLOGY, Volume 148, No 5, March 1992, Bodo Ortmann et al, "Syntetic peptides anchor T cell-specific TNP epitopes to MHC" page 1445 - page 1450 --	1,6,7
X	TETRAHEDRON LETTERS, Volume 32, No 51, 1991, Mikael Elofsson et al, "Building Blocks for Glycopeptide Synthesis: Glycosylation of 3-Mercaptopropionic Acid and Fmoc Amino Acids with Unprotected Carboxyl Groups" page 7613 - page 7616 --	38
Y	WO, A2, 8906974 (PRAXIS BIOLOGICS, INC.), 10 August 1989 (10.08.89), page 25 - page 27; page 29 - page 31 --	1-17,19,20, 22-24,26-32
Y	EP, A1, 0427347 (ENIRICERCHE S.P.A.), 15 May 1991 (15.05.91), page 3, line 57, claim 2 -- -----	1-17,19,20, 22-24,26-32

INTERNATIONAL SEARCH REPORT

Information on patent family members

30/07/93

International application No.

PCT/SE 93/00353

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A1- 8907448	24/08/89	AU-A- 4030989 EP-A- 0400077	06/09/89 05/12/90
WO-A2- 8906974	10/08/89	AU-B- 634153 AU-A- 3065489 EP-A- 0399001	18/02/93 25/08/89 28/11/90
EP-A1- 0427347	15/05/91	US-A- 5196512	23/03/93