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(54) **Titre : UTILISATION DE L'ADENOSINE DESAMINASE POUR LA THERAPIE CONTRE LE CANCER**  
(54) **Title: USE OF ADENOSINE DEAMINASE FOR CANCER THERAPY**

(57) **Abrégé/Abstract:**

What is provided is a method of treating a patient having a tumor comprising administering an effective amount of adenosine deaminase, preferably polyalkylene oxide conjugated, such as adenosine deaminase conjugated to polyethylene glycol, to a patient in need thereof.



ABSTRACT

What is provided is a method of treating a patient having a tumor comprising administering an effective amount of adenosine deaminase, preferably polyalkylene oxide conjugated, such as adenosine deaminase conjugated to polyethylene glycol, to a patient in need thereof.

## USE OF ADENOSINE DEAMINASE FOR CANCER THERAPY

5

### FIELD OF THE INVENTION

10           The invention provides new methods of treating and inhibiting tumors, and especially malignant solid tumors, by administering adenosine deaminase in order to reduce tissue levels of adenosine and deoxyadenosine.

### BACKGROUND OF THE INVENTION

15           A tumor is an abnormal benign or malignant growth of cells or tissue that arises from uncontrolled cellular proliferation. A malignant tumor is a one that spreads from its site of origin, and is also art-known as a cancer. Thus, tumors and cancers are a family of diseases sharing the common property of uncontrolled or inappropriate cell growth. Broadly, malignant tumors are either blood derived tumors, such as leukemia, or solid tumors. Blood  
20           derived malignant tumors generally circulate in the blood, but solid malignant tumors spread throughout the body from a primary tumor. The distributed tumor cells then have the potential to develop into multiple secondary tumors, in a process of metastasis. In order for a solid tumor to undergo such a metastatic spread, solid tumor cells must escape from the primary or original tumor, enter the blood stream or lymphatic system, and from there invade  
25           the tissue of other organs, where they multiply and form new tumors. Metastasis is a complex multi-step process that involves changes in tumor cell adhesion and motility, secretion of proteolytic enzymes, chemoattractants, and proteoglycans and other factors. In addition, angiogenesis, or the formation of new blood vessels, is also a vital step in the metastatic process (Folkman, 1995, *Nature Medicine* 1:27-31).

30           The immune system has also been shown to inhibit the metastasis of such malignant tumor cells, and it has been reported that adenosine, in turn, may inhibit such immune

protective reactions. For example, Loshkin et al., (2006, *Cancer Res.* 66: 7758-7765) report that adenosine inhibits activation and cytokine production in killer T cells. Adenosine negatively impacts other immune function, including both cellular elements and inflammatory functions (see, e.g., the reviews by Szychala, 2000, *Pharmacology & Therapeutics* 87: 161–  
5 173 and by Sitkovesky et al., 2005 *Nature Reviews Immunology* 5: 713-721). Sitkovesky et al., in WO 03/050241, published June 19, 2003, also described methods for increasing an immune response to an antigen and for treating tumors, by administering an adenosine receptor antagonist, that can include adenosine deaminase.

It has also been shown that adenosine promotes tumor cell migration and angiogenesis  
10 (Barcz et al., 2000, *Oncol. Rep.* 7(6): 1285-91; Adair, 2005, *Am J Physiol Regul Integr Comp Physiol* 289: R283–R296) and that adenosine stimulates the proliferation of colon cancer cells (Mujoomdar et al. 2003, *Biochemical Pharmacology* 66 1737–1747). It has also been reported by Asmar et al., 1966, *Proc. Am. Assoc. Cancer Res.* (Abstract No. 73) that the  
15 growth of certain tumor cells was inhibited, by injection of ADA, by over 50% in a mouse ascites model. These were lymphatic leukemias L1210 and L4946, lymphosarcoma 6C3HED, mammary adenocarcinoma TA3, and Ehrlich carcinoma E2. In the same Abstract, an adenocarcinoma 755 was reported to be twice as resistant to and a sarcoma 180 was completely resistant to the effect. WO03050241A2 describes the effects of an inhibitor of  
20 adenosine receptors on B16 melanoma cells. While WO03050241A2 mentions ADA as an inhibitor of adenosine, there is no specific description for applying ADA to the treatment of specific cancers, and particularly ovarian cancer and prostate cancer.

Thus, it seems that for some tumors, the presence of adenosine provides a “go” signal for tumor proliferation and for tumor angiogenesis, and a “stop” signal for the killer T cells which would normally kill these tumors.

25 In contrast to the above-discussed findings, Lind, et al. (U.S. Patent No. 6,579,857) have reported that adenosine, in combination with an inhibitor of the enzyme adenosine deaminase, and/or in combination with an anticancer agent such as coformycin, is useful in a method for potentiating cell death in neoplastic cells of epithelial origin. Thus, this reference suggests that the role of adenosine in cancer is more complex and unsettled.

30 As noted above, an agent for reducing endogenous adenosine levels is the enzyme adenosine deaminase. Adenosine deaminase ("ADA"), designated as EC 3.5.4.4, is an



important enzyme of the purine salvage pathway. ADA converts either adenosine or deoxyadenosine, in the presence of water, into inosine or deoxyinosine and ammonia. It is known that individuals who harbor deleterious mutations in the ADA gene can develop varying degrees of an immunodeficiency disorder, from mild to severe, i.e. severe combined  
5 immunodeficiency disorder ("SCID"). SCID has been confirmed to result from the toxic accumulation of the enzyme substrates, adenosine and deoxyadenosine, in immature lymphoid cells. The onset of the disorder can range from early childhood to adults, depending on the mutations inherited. Deficiencies of ADA are one of the leading causes of SCID, in children, and is one of the leading targets for gene therapy approaches (R. Parkman et al.,  
10 2000, "Gene therapy for adenosine deaminase deficiency", *Ann. Rev. Med.*, 51:33-47).

Previously, ADA has been commercially isolated from bovine sources and employed in treating a number of disorders, including SCID, in the form of a bovine ADA conjugated to polyethylene glycol ("PEG") polymer. PEGylated ADA for medical use is commercially available from Enzon Pharmaceuticals, Inc. as ADAGEN<sup>®</sup>, PEGylated ADA. The  
15 conjugation of a PEG moiety to ADA allows the enzyme to achieve its full therapeutic effect by increasing the circulating life and rendering the ADA substantially non-antigenic, in order to minimize the potential for immunogenic reactions. It is also possible to produce recombinant human or bovine ADA enzymes for use in a conjugated form, as described by U.S. Patent No. 8,071,741, entitled "Stable Recombinant Adenosine Deaminase".  
20

Thus, there is a longstanding need in the art for new and improved methods of treating  
25 or inhibiting the growth, spread and development of cancers.

### SUMMARY OF THE INVENTION

Accordingly, the invention provides a method of treating a patient having a tumor comprising administering an effective amount of ADA to the patient in need thereof. An  
30 effective amount is that which is readily determined by one of ordinary skill in the art to reduce tissue levels of adenosine or deoxyadenosine in the patient, and wherein growth or

spread of the tumor is inhibited by substantially reduced tissue levels of adenosine in the patient. The route of administration is a route such as subcutaneous, intravenous, intramuscular, intrathecal, intraperitoneal, inhalation and transurethral.

5 The tumor can be malignant or nonmalignant, and is preferably a solid tumor, e.g., a tumor such as a prostate tumor, an ovarian cancer and/or a colorectal cancer.

The adenosine deaminase is preferably conjugated to a substantially non-antigenic polymer, such as a polyalkylene oxide (PAO). The PAO preferably ranges in size from about 4,000 to about 45,000 Daltons. The PAO is preferably a polyethylene glycol ("PEG"). The molar ratio of ADA to polymer can be 1:1, or can be two or more ADA molecules per  
10 polymer, or more preferably, provides for from about 1 to about 20 polymer molecules (i.e. 11-18 PEG strands) per ADA molecule.

The polymer-conjugated ADA is preferably administered in a dose ranging from about 10U to about 30U per kg or more and for a sufficient period of time to maintain inhibition of the tumor, e.g., from about 1 to about 20 days, or longer.

15 The amount of adenosine deaminase that is administered is effective to substantially reduce tissue levels of adenosine or deoxyadenosine in the patient, and wherein growth or spread of the tumor is inhibited by substantially reduced tissue levels of adenosine in said patient. This is, for example, a dose of adenosine deaminase ranging from about 10U to about 30U per kg. The dose is repeated for a sufficient period of time to maintain inhibition of the  
20 tumor, e.g., from about 1 to about 20 days, or longer. The dose is administered by any convenient route such as subcutaneous, intravenous, intramuscular, intrathecal, intraperitoneal, inhalation and transurethral.

The adenosine deaminase is optionally purified from a bovine source or is a recombinant adenosine deaminase. The recombinant adenosine deaminase is, for example,  
25 recombinant bovine adenosine deaminase (Ser74-rbADA) comprising SEQ ID NO: 1, recombinant human adenosine deaminase (Ser74-rhADA) comprising SEQ ID NO: 3 and recombinant bovine adenosine deaminase comprising SEQ ID NO: 5 and/ or variations or polymorphisms thereof. Recombinantly produced bovine adenosine deaminase, e.g., SEQ ID NO: 5, is optionally capped at Cys74 for stability in an aqueous medium.



For purposes of the present invention, the term "residue" shall be understood to mean that portion of a compound, to which it refers, e.g., PEG, ADA, amino acid, etc. that remains after it has undergone a substitution reaction with another compound.

For purposes of the present invention, the term "polymeric residue", e.g., "PEG residue", shall each be understood to mean that portion of the polymer or PEG which remains after it has undergone a reaction with other compounds, moieties, etc.

For purposes of the present invention, the term "alkyl" as used herein refers to a saturated aliphatic hydrocarbon, including straight-chain, branched-chain, and cyclic alkyl groups. The term "alkyl" also includes alkyl-thio-alkyl, alkoxyalkyl, cycloalkylalkyl, heterocycloalkyl, and C<sub>1-6</sub> alkylcarbonylalkyl groups. Preferably, the alkyl group has 1 to 12 carbons. More preferably, it is a lower alkyl of from about 1 to 7 carbons, yet more preferably about 1 to 4 carbons. The alkyl group can be substituted or unsubstituted. When substituted, the substituted group(s) preferably include halo, oxy, azido, nitro, cyano, alkyl, alkoxy, alkyl-thio, alkyl-thio-alkyl, alkoxyalkyl, alkylamino, trihalomethyl, hydroxyl, mercapto, hydroxy, cyano, alkylsilyl, cycloalkyl, cycloalkylalkyl, heterocycloalkyl, heteroaryl, alkenyl, alkynyl, C<sub>1-6</sub> hydrocarbonyl, aryl, and amino groups.

For purposes of the present invention, the term "substituted" as used herein refers to adding or replacing one or more atoms contained within a functional group or compound with one of the moieties from the group of halo, oxy, azido, nitro, cyano, alkyl, alkoxy, alkyl-thio, alkyl-thio-alkyl, alkoxyalkyl, alkylamino, trihalomethyl, hydroxyl, mercapto, hydroxy, cyano, alkylsilyl, cycloalkyl, cycloalkylalkyl, heterocycloalkyl, heteroaryl, alkenyl, alkynyl, C<sub>1-6</sub> alkylcarbonylalkyl, aryl, and amino groups.

For purposes of the present invention, the term "alkenyl" refers to groups containing at least one carbon-carbon double bond, including straight-chain, branched-chain, and cyclic groups. Preferably, the alkenyl group has about 2 to 12 carbons. More preferably, it is a lower alkenyl of from about 2 to 7 carbons, yet more preferably about 2 to 4 carbons. The alkenyl group can be substituted or unsubstituted. When substituted the substituted group(s) preferably include halo, oxy, azido, nitro, cyano, alkyl, alkoxy, alkyl-thio, alkyl-thio-alkyl, alkoxyalkyl, alkylamino, trihalomethyl, hydroxyl, mercapto, hydroxy, cyano, alkylsilyl, cycloalkyl, cycloalkylalkyl, heterocycloalkyl, heteroaryl, alkenyl, alkynyl, C<sub>1-6</sub> hydrocarbonyl, aryl, and amino groups.

For purposes of the present invention, the term "alkynyl" refers to groups containing at least one carbon-carbon triple bond, including straight-chain, branched-chain, and cyclic groups. Preferably, the alkynyl group has about 2 to 12 carbons. More preferably, it is a lower alkynyl of from about 2 to 7 carbons, yet more preferably about 2 to 4 carbons. The  
5 alkynyl group can be substituted or unsubstituted. When substituted the substituted group(s) preferably include halo, oxy, azido, nitro, cyano, alkyl, alkoxy, alkyl-thio, alkyl-thio-alkyl, alkoxyalkyl, alkylamino, trihalomethyl, hydroxyl, mercapto, hydroxy, cyano, alkylsilyl, cycloalkyl, cycloalkylalkyl, heterocycloalkyl, heteroaryl, alkenyl, alkynyl, C<sub>1-6</sub> hydrocarbonyl, aryl, and amino groups. Examples of "alkynyl" include propargyl,  
10 propyne, and 3-hexyne.

For purposes of the present invention, the term "aryl" refers to an aromatic hydrocarbon ring system containing at least one aromatic ring. The aromatic ring can optionally be fused or otherwise attached to other aromatic hydrocarbon rings or non-aromatic hydrocarbon rings. Examples of aryl groups include, for example, phenyl, naphthyl, 1,2,3,4-  
15 tetrahydronaphthalene and biphenyl. Preferred examples of aryl groups include phenyl and naphthyl.

For purposes of the present invention, the term "cycloalkyl" refers to a C<sub>3-8</sub> cyclic hydrocarbon. Examples of cycloalkyl include cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl and cyclooctyl.

20 For purposes of the present invention, the term "cycloalkenyl" refers to a C<sub>3-8</sub> cyclic hydrocarbon containing at least one carbon-carbon double bond. Examples of cycloalkenyl include cyclopentenyl, cyclopentadienyl, cyclohexenyl, 1,3-cyclohexadienyl, cycloheptenyl, cycloheptatrienyl, and cyclooctenyl.

25 For purposes of the present invention, the term "cycloalkylalkyl" refers to an alkyl group substituted with a C<sub>3-8</sub> cycloalkyl group. Examples of cycloalkylalkyl groups include cyclopropylmethyl and cyclopentylethyl.

30 For purposes of the present invention, the term "alkoxy" refers to an alkyl group of indicated number of carbon atoms attached to the parent molecular moiety through an oxygen bridge. Examples of alkoxy groups include, for example, methoxy, ethoxy, propoxy and isopropoxy.



For purposes of the present invention, an “alkylaryl” group refers to an aryl group substituted with an alkyl group.

For purposes of the present invention, an “aralkyl” group refers to an alkyl group substituted with an aryl group.

5 For purposes of the present invention, the term “alkoxyalkyl” group refers to an alkyl group substituted with an alkoxy group.

For purposes of the present invention, the term “alkyl-thio-alkyl” refers to an alkyl-S-alkyl thioether, for example methylthiomethyl or methylthioethyl.

10 For purposes of the present invention, the term “amino” refers to a nitrogen containing group as is known in the art derived from ammonia by the replacement of one or more hydrogen radicals by organic radicals. For example, the terms “acylamino” and “alkylamino” refer to specific N-substituted organic radicals with acyl and alkyl substituent groups respectively.

15 For purposes of the present invention, the term “alkylcarbonyl” refers to a carbonyl group substituted with alkyl group.

For purposes of the present invention, the terms “halogen” or “halo” refer to fluorine, chlorine, bromine, and iodine.

20 For purposes of the present invention, the term “heterocycloalkyl” refers to a non-aromatic ring system containing at least one heteroatom selected from nitrogen, oxygen, and sulfur. The heterocycloalkyl ring can be optionally fused to or otherwise attached to other heterocycloalkyl rings and/or non-aromatic hydrocarbon rings. Preferred heterocycloalkyl groups have from 3 to 7 members. Examples of heterocycloalkyl groups include, for example, piperazine, morpholine, piperidine, tetrahydrofuran, pyrrolidine, and pyrazole. Preferred heterocycloalkyl groups include piperidinyl, piperazinyl, morpholinyl, and  
25 pyrrolidinyl.

For purposes of the present invention, the term “heteroaryl” refers to an aromatic ring system containing at least one heteroatom selected from nitrogen, oxygen, and sulfur. The heteroaryl ring can be fused or otherwise attached to one or more heteroaryl rings, aromatic or non-aromatic hydrocarbon rings or heterocycloalkyl rings. Examples of heteroaryl groups  
30 include, for example, pyridine, furan, thiophene, 5,6,7,8-tetrahydroisoquinoline and pyrimidine. Preferred examples of heteroaryl groups include thienyl, benzothienyl, pyridyl,

quinolyl, pyrazinyl, pyrimidyl, imidazolyl, benzimidazolyl, furanyl, benzofuranyl, thiazolyl, benzothiazolyl, isoxazolyl, oxadiazolyl, isothiazolyl, benzisothiazolyl, triazolyl, tetrazolyl, pyrrolyl, indolyl, pyrazolyl, and benzopyrazolyl.

For purposes of the present invention, the term “heteroatom” refers to nitrogen,  
5 oxygen, and sulfur.

In some embodiments, substituted alkyls include carboxyalkyls, aminoalkyls, dialkylaminos, hydroxyalkyls and mercaptoalkyls; substituted alkenyls include carboxyalkenyls, aminoalkenyls, dialkenylaminos, hydroxyalkenyls and mercaptoalkenyls; substituted alkynyls include carboxyalkynyls, aminoalkynyls, dialkynylaminos,  
10 hydroxyalkynyls and mercaptoalkynyls; substituted cycloalkyls include moieties such as 4-chlorocyclohexyl; aryls include moieties such as naphthyl; substituted aryls include moieties such as 3-bromo phenyl; aralkyls include moieties such as tolyl; heteroalkyls include moieties such as ethylthiophene; substituted heteroalkyls include moieties such as 3-methoxy-thiophene; alkoxy includes moieties such as methoxy; and phenoxy includes  
15 moieties such as 3-nitrophenoxy. Halo shall be understood to include fluoro, chloro, iodo and bromo.

For purposes of the present invention, “positive integer” shall be understood to include an integer equal to or greater than 1 and as will be understood by those of ordinary skill to be within the realm of reasonableness by the artisan of ordinary skill.

For purposes of the present invention, the term “linked” shall be understood to include  
20 covalent (preferably) or noncovalent attachment of one group to another, i.e., as a result of a chemical reaction.

The terms “effective amounts” and “sufficient amounts” for purposes of the present invention shall mean an amount which achieves a desired effect or therapeutic effect as such  
25 effect is understood by those of ordinary skill in the art.

For purposes of the present invention, the term “adenosine” shall be understood to include the nucleosides adenosine and deoxyadenosine. Adenosine also includes adenosine and deoxyadenosine present in the form of AMP, ADP, ATP, dAMP, dADP or dATP.

For purposes of the present invention, “adenosine-mediated tumor” or “adenosine  
30 deaminase-responsive tumor” shall be understood as broadly including any types of tumors



which benefit from the administration of ADA, or active fraction thereof, etc., regardless of the route of administration.

For purposes of the present invention, “treatment of adenosine-mediated tumor” or “treatment of adenosine deaminase-responsive tumor” or “inhibition of adenosine-mediated tumor growth” or “inhibition of adenosine deaminase-responsive tumor growth” shall be understood to mean that symptoms or conditions are minimized or attenuated when compared to that observed in the absence of the ADA treatment. The treated conditions can be confirmed by, for example, tumor growth inhibition and/or decrease in adenosine levels in cancer cells or tissues.

Broadly speaking, successful treatment shall be deemed to occur when the desired clinical response is obtained. For example, successful treatment can be defined by obtaining e.g, 10% or higher (i.e. 20% 30%, 40%) tumor growth inhibition. Alternatively, successful treatment can be defined by obtaining at least 20% or preferably 30%, more preferably 40 % or higher (i.e., 50% or 80%) decrease in adenosine levels in cancer cells or tissues, including other clinical markers contemplated by the artisan in the field, when compared to that observed in the absence of the ADA treatment described herein.

Further, the use of singular terms for convenience in description is in no way intended to be so limiting. Thus, for example, reference to a composition comprising an enzyme refers to one or more molecules of that enzyme. It is also to be understood that this invention is not limited to the particular configurations, process steps, and materials disclosed herein as such configurations, process steps, and materials may vary somewhat.

It is also to be understood that the terminology employed herein is used for the purpose of describing particular embodiments only and is not intended to be limiting, since the scope of the present invention will be limited by the appended claims and equivalents thereof.

### **DETAILED DESCRIPTION OF THE INVENTION**

Accordingly, the invention provides for new methods of treating tumors, including cancers, by administering ADA enzyme to a patient in need thereof, in an amount, and for a duration sufficient to reduce the amount of adenosine present in the tissues and/or body fluids of the patient. Preferably, the ADA enzyme is polymer conjugated. For those tumors that



depend upon the presence of adenosine for growth, metastatic spread and/or for protection from the patient's immune system, a sufficient reduction in endogenous adenosine will limit or prevent cancer growth, metastatic spread and/or allow the normal operation of antitumor activity by the patient's immune system.

5 It is also contemplated that the inventive ADA treatment of tumors is optionally conducted in combination or coordination with one or more other appropriate art-known anti-cancer treatment methods and agents, as discussed in greater detail herein below. The combination treatment of the present invention includes administering an effective amount of the compounds described herein alone or in combination, simultaneously or sequentially, with  
10 a second chemotherapeutic agent.

Exemplary art-known anti-cancer agents for such combination treatments include, e.g., Taxol™, bevacizumab (Avastin®), vincristine, vinblastine, neomycin, combretastatin(s), podophyllotoxin(s), TNF-alpha, angiostatin, endostatin, vasculostatin, alpha<sub>v</sub>-beta<sub>3</sub> antagonists, calcium ionophores, calcium-flux inducing agents, any derivative or prodrug  
15 thereof.

The additional anti-cancer agents also include chemotherapeutic agents, radiotherapeutic agents, cytokines, anti-angiogenic agents, apoptosis-inducing agents or anti-cancer immunotoxins or coagulgands. One such immunotoxin is e.g. Erbitux® (cetuximab). "Chemotherapeutic agents", as used herein, refer to classical chemotherapeutic agents or  
20 drugs used in the treatment of malignancies. This term is used for simplicity notwithstanding the fact that other compounds may be technically described as chemotherapeutic agents in that they exert an anti-cancer effect. However, "chemotherapeutic" has come to have a distinct meaning in the art and is being used according to this standard meaning.

Thus, the inventive method can be employed in combination with one or more  
25 chemotherapeutic agents known to be effective in treating cancer, including but not limited to, 5-azacytidine, 5-flourouracil, optionally in combination with leucovorin, 5-fluorodeoxyuridine, 6-mercaptopurine, 6-thioguanine, mitoxantrone, aziridinybenzoquinone (AZQ), Carmustine (BCNU or BiCNU® from Bristol-Myers Squibb), bleomycin, carboplatin (CBDCA), Lomustine (CCNU), methyl-CCNU or MeCCNU,  
30 chlorambucil, chlorodeoxyadenosine, cisplatin, cyclophosphamide, cytarabine, dactinomycin, daunorubicin, deoxycoformycin, doxorubicin, doxycoformycin, DTIC (dacarbazine),

epirubicin, etoposide (VP-16), fludarabine, hexamethylmelamine, hydroxyurea, idarubicin, ifosfamide, ifosfamide and mesna, levamisol, N-acetylcysteine ("NAC"), l-phenylalanine mustard, 4'-(9-acridinylamino)methanesulfon-m-anisidide ("mAMSA"), art-known inhibitors of multiple drug resistance (ie., MDR inhibitors), melphalan, methotrexate, optionally in  
5 combination with leucovorin, mithramycin, mitomycin-c, inhibitors of multidrug resistance related protein ("MRP" inhibitors), paclitaxel, procarbazine, streptozotocin, N,N,N'-triethylenethiophosphoramidate ("thioTEPA"), inhibitors of topoisomerase I and/or topoisomerase II, taxol, vinblastine, vincristine, vindesine, teniposide (VM-26<sup>®</sup>), and others too numerous to mention.

10 Other methods of treating cancer contemplated to be employed in combination with the methods of the invention include irradiation with X-rays, gamma rays whether directly or with tomographic targeting, treatment of cancerous tissues with implanted radioactive pellets or "seeds," neutron beam irradiation of tissues primed with boron compounds, and/or other types of art-known particle beam therapy.

15 Further antitumor or anticancer agents (antineoplastic agents) that are optionally administered in combination or coordination with the instant inventive methods include those described by "GOODMAN AND GILMAN'S, THE PHARMACOLOGICAL BASIS OF THERAPEUTICS", TENTH EDITION, Eds. Hardman and Limbird.

20 In order to better appreciate the invention, the following terms are defined.

Reference to "adenosine" herein also encompasses deoxyadenosine, and art-known variations and derivatives thereof present *in vivo*, unless otherwise specified.

Reference to adenosine deaminase or ADA includes any art-known form of the enzyme, including purified natural enzyme, e.g., purified natural ADA, recombinant human or  
25 bovine ADA and variations, polymorphisms, and derivatives thereof. ADA enzyme purified from bovine sources has a sequence according to SEQ ID NO: 5, with the Cys 74 residue naturally capped or protected by a cysteine, and the six C-terminal residues predicted from the gene encoding the ADA of SEQ ID NO: 5 are not present. However, it is contemplated that the invention can be practiced with alternative variations on natural bovine ADA, including  
30 alternative alleles and polymorphisms, both natural and recombinantly produced, with and without the predicted six C-terminal residues. Bovine ADA polymorphisms include, e.g.,



glutamine at position 198 in place of lysine; alanine at position 245 in place of threonine; arginine at position 351 instead of glycine.

Preferred derivatives of ADA enzyme include recombinantly produced ADA enzyme that has been mutated for enhanced stability relative to nonmutated recombinant ADA enzyme. These include, for example, recombinant ADA enzymes modified from SEQ ID NO: 5 and/or SEQ ID NO: 5 with one or more of the above-noted polymorphisms, to replace an oxidizable Cys residue that is solvent-exposed with a suitable non-oxidizable amino acid residue. Such non-oxidizable residue includes any art-known natural amino acid residue and/or any art-known derivatives thereof. For example, mature recombinant ADA expressed from a gene first isolated from a bovine or human source has an unstable Cys<sub>74</sub> residue that is preferably replaced with a non-oxidizable amino acid residue, e.g., by a Ser<sub>74</sub>. Such recombinant ADA enzymes are illustrated by SEQ ID NO: 1 (bovine ADA structure) and SEQ ID NO: 3 (human ADA structure). SEQ ID NO: 2 and SEQ ID NO: 4 illustrate useful DNA molecules for expressing the same, that have been codon optimized for *E. coli* expression. Additional details concerning such recombinant ADA muteins, and production and purification of these proteins, are provided by co-owned U.S. Patent No. 8,071,741, entitled "Stable Recombinant Adenosine Deaminase" filed on even date herewith.

Specific details on the vectors and methods of purification are found therein, particularly in the Examples section, and most particularly in Examples 1-4.

Alternatively, ADA enzymes can be stabilized, as needed, by capping a solvent-exposed oxidizable Cys residue, by treating the ADA enzyme with a sufficient amount of a capping agent, under reaction conditions sufficient to cap the reactive cysteine, without substantially inactivating the ADA protein. This process is preferably employed with recombinantly produced ADA, either mutein or wild-type ADA enzymes.

For example, capping agents include, without limitation, oxidized glutathione (preferred), iodoacetamide, iodoacetic acid, cystine, and other dithiols known to those of ordinary skill, and mixtures thereof. The amount and concentration of the capping agent included during the reacting phase of the methods described herein will depend somewhat upon the specific capping agent used and the needs of the artisan but will not be subject to



undue experimentation. Using oxidized glutathione as a prototype, the concentration of used when reacted with the recombinant protein such as rhADA can range from about 25  $\mu$ M to about 100 mM. Preferably, the oxidized glutathione is reacted with the recombinant protein at a concentration of from about 5 nM to about 25 mM.

5           The reaction conditions employed during the reacting of the capping agent and the recombinant protein further include the use of an aqueous solution having a pH of from about 6.5 to about 8.4, preferably from about 7.2 to about 7.8. In addition, the aqueous solution preferably includes a suitable buffer such as sodium phosphate, potassium phosphate, Tris, and Hepes and mixtures thereof at concentrations ranging from 10 to 150 mM (optionally,  
10       capping can take place out of this buffer range, lower than 10 or high than 150 mM). The reaction conditions further include allowing the reaction to proceed at temperatures which will not contribute to degradation of the protein, i.e. from about 4 -37° C. Optionally, capping can take place outside of this temperature range, e.g., at a temperature range lower than 0-4 °C or higher than 37°C. The reaction is conducted for a time sufficient to achieve the desired  
15       stabilization of the reactive cysteine. Simply by way of example, the reaction is conducted for a time ranging from about 5 seconds to about 8 hours (e.g., overnight).

20           Further, the use of singular terms for convenience in description is in no way intended to be so limiting. Thus, for example, reference to a composition comprising an enzyme refers to one or more molecules of that enzyme. It is also to be understood that this invention is not limited to the particular configurations, process steps, and materials disclosed herein as such configurations, process steps, and materials may vary somewhat.

25

#### A. Polymer-ADA Conjugates

A preferred form of ADA is in the form of a polymer conjugated enzyme. The ADA-polymer conjugates of the present invention generally correspond to formula (I):



30

wherein

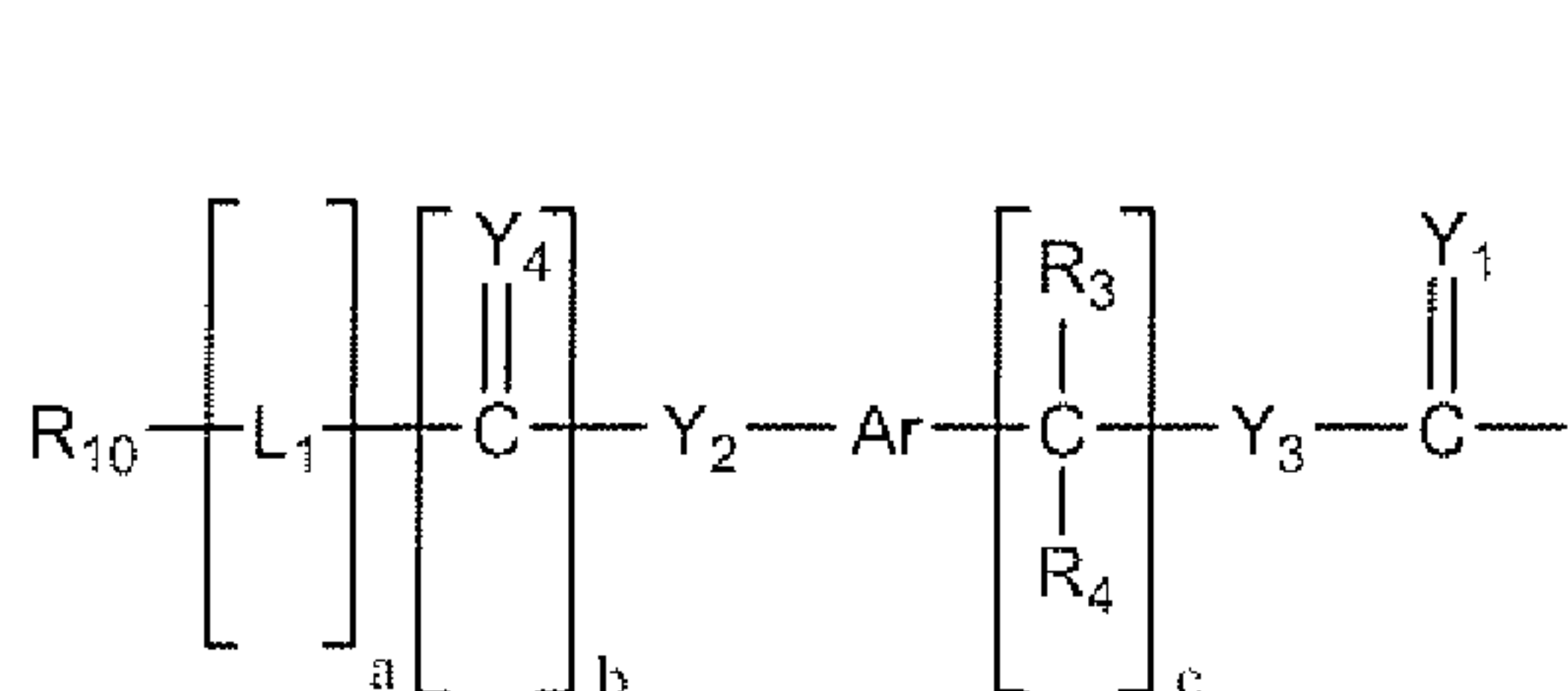
(ADA) represents the adenosine deaminase enzyme or optionally, a derivative or fragment thereof;

NH- is an amino group of an amino acid found on the ADA, derivative or fragment thereof for attachment to the polymer;

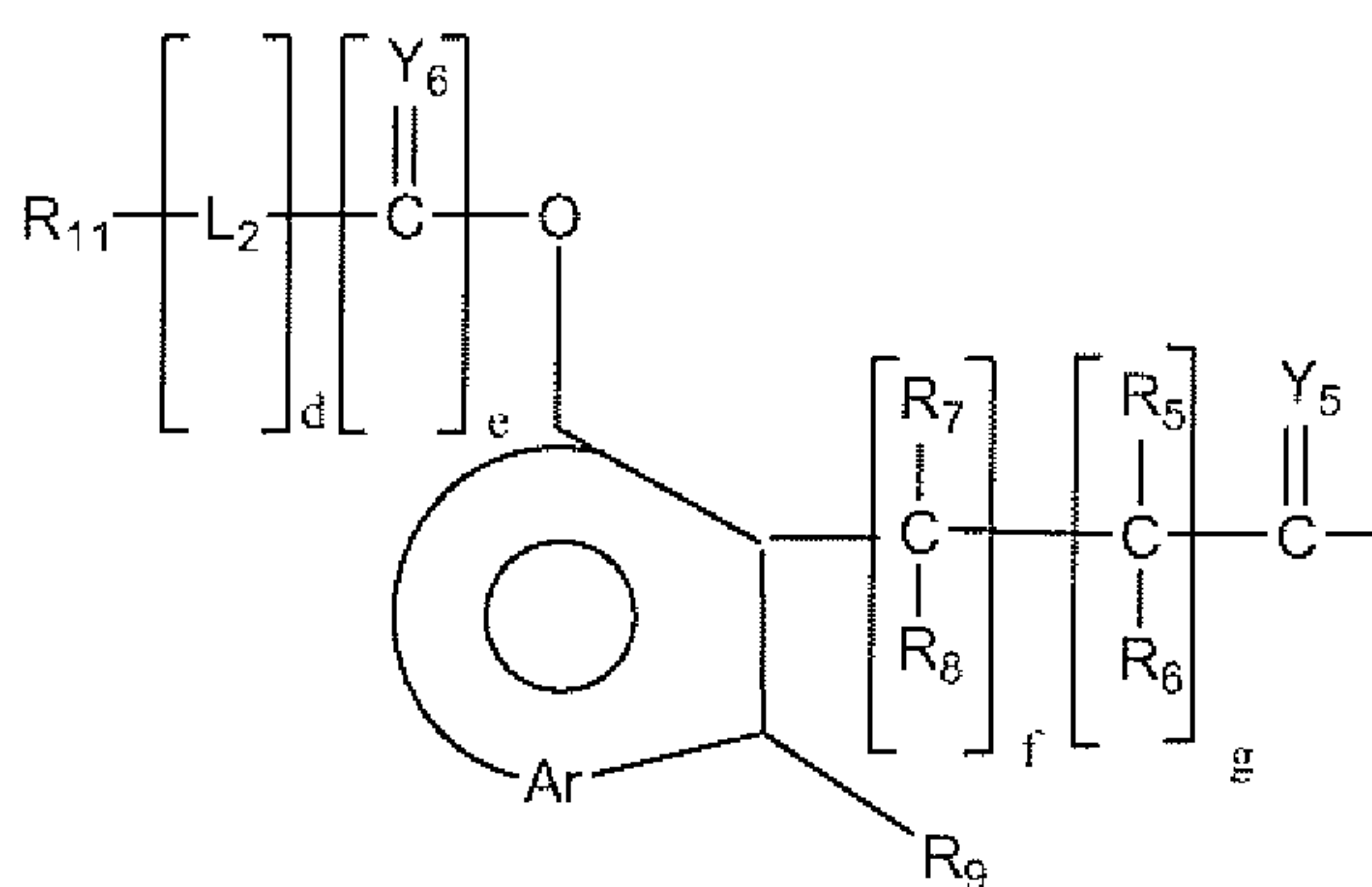
5 (z) is a positive integer, preferably from about 1 to about 80, more preferably from about 5 to about 80, yet more preferably from about 11 to about 18; and

R is a substantially non-antigenic polymer residue that is attached to the ADA in a releasable or non-releasable form.

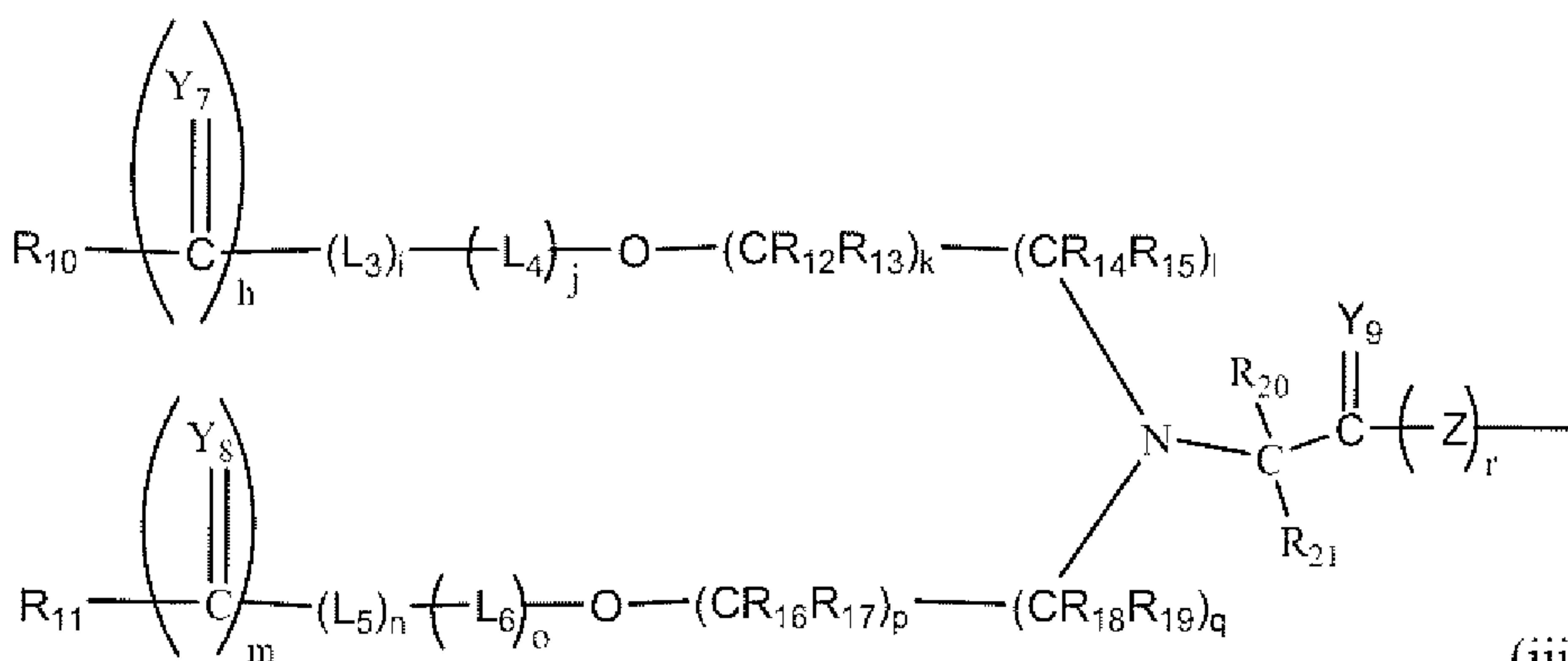
10 The non-antigenic polymer residue portion of the conjugate (R) can be selected from among a non-limiting list of polymer-based systems such as:



(i)



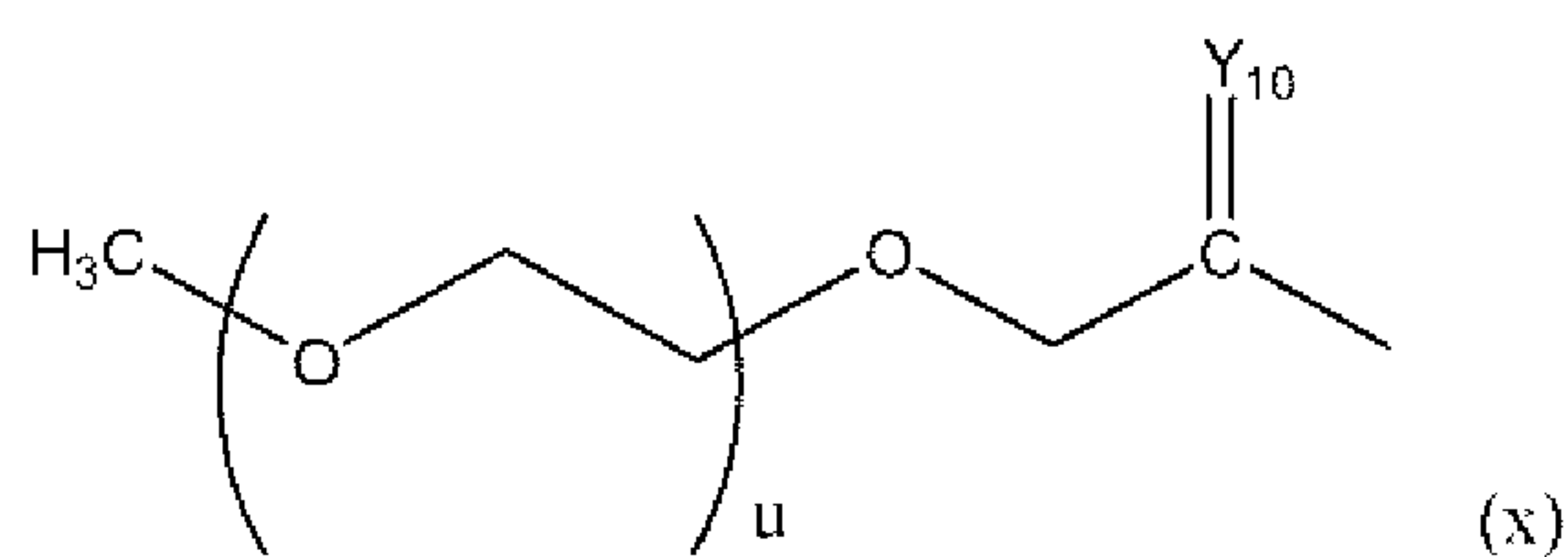
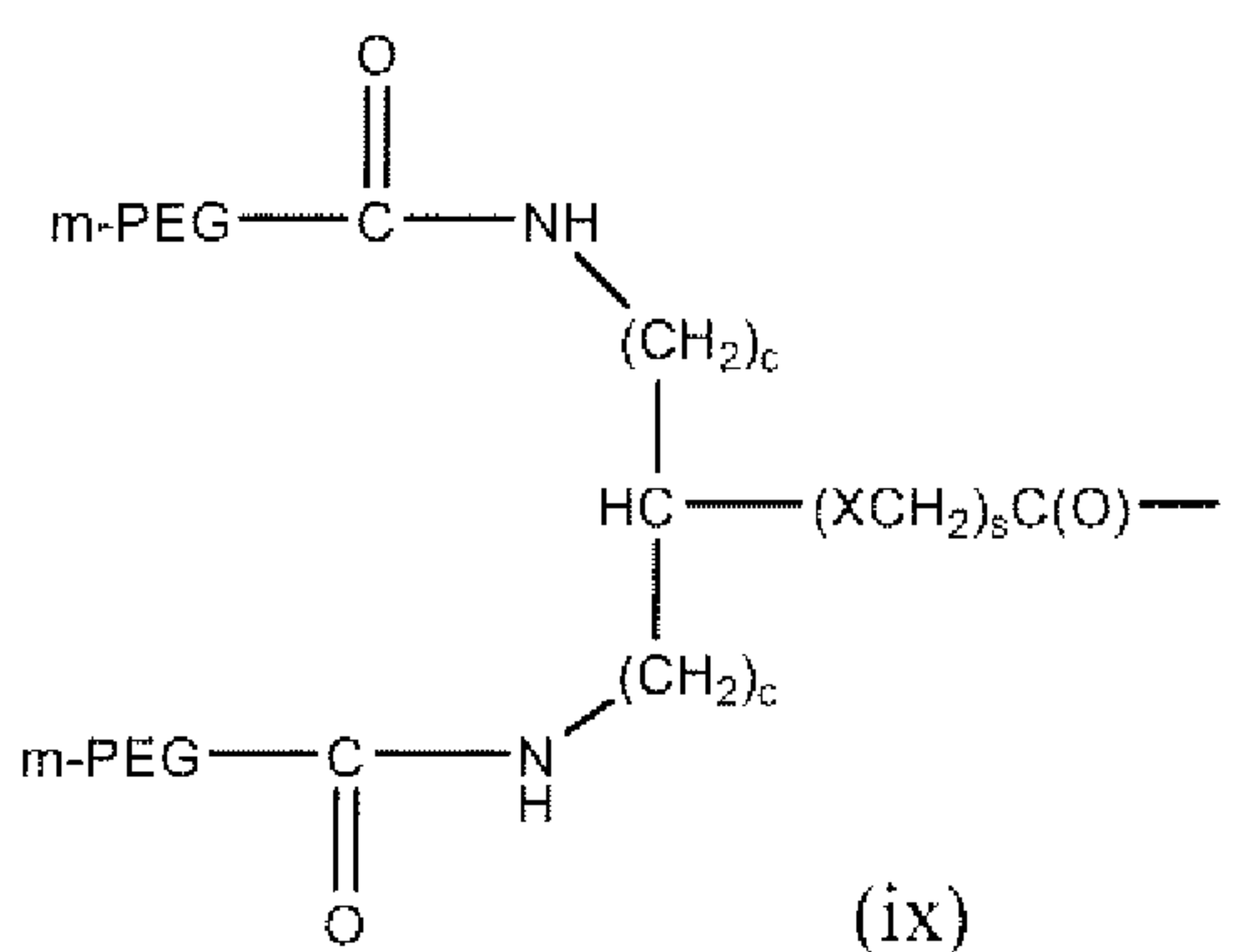
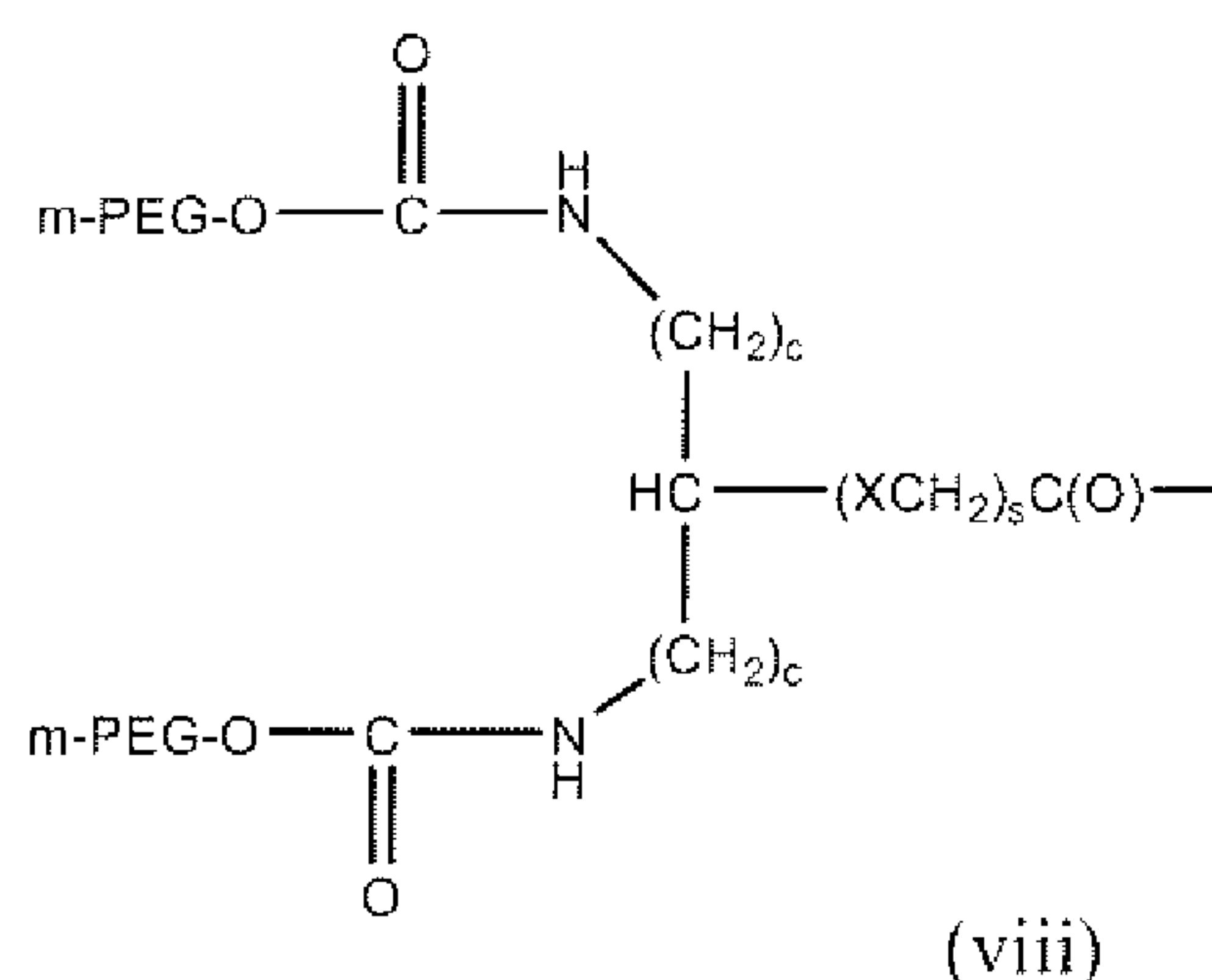
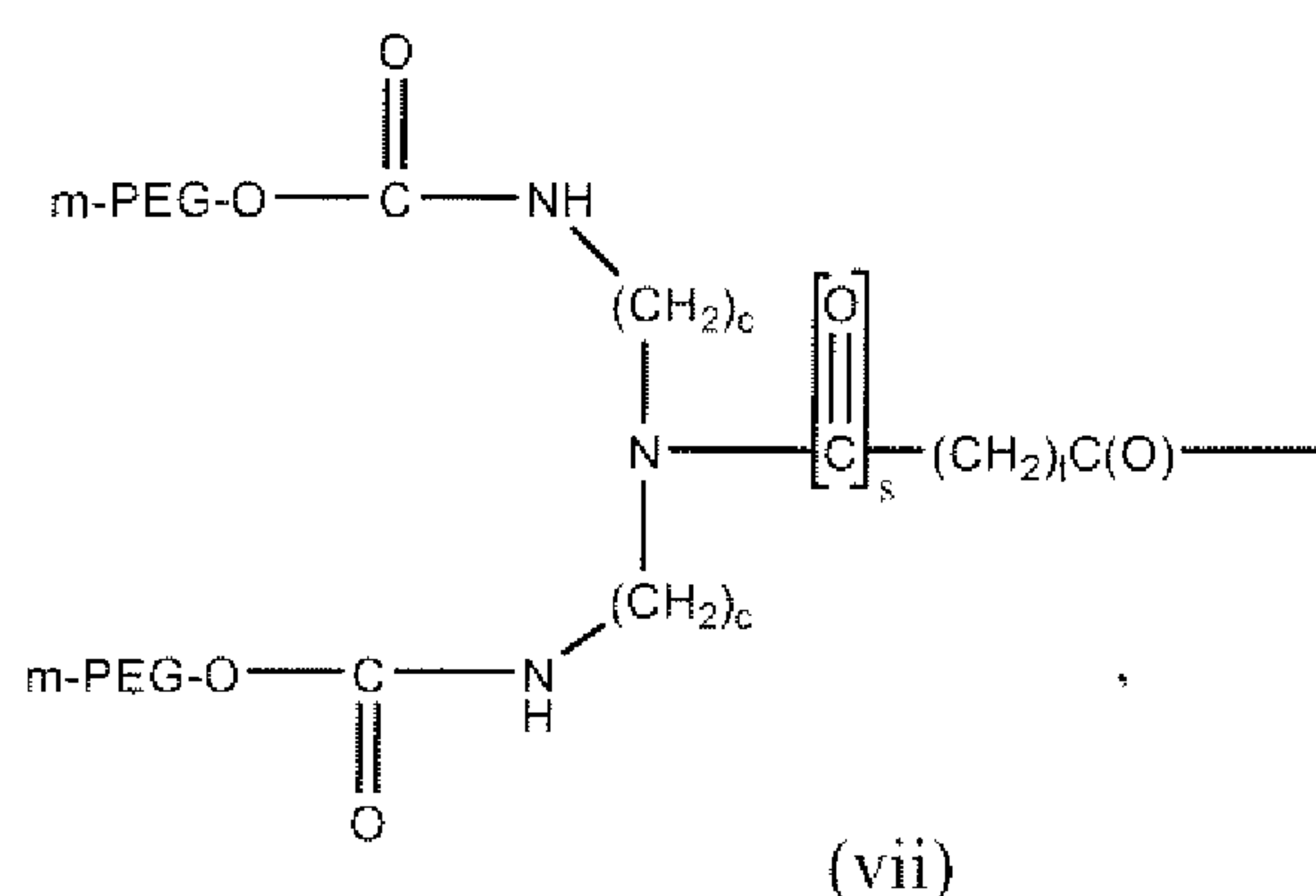
(ii)



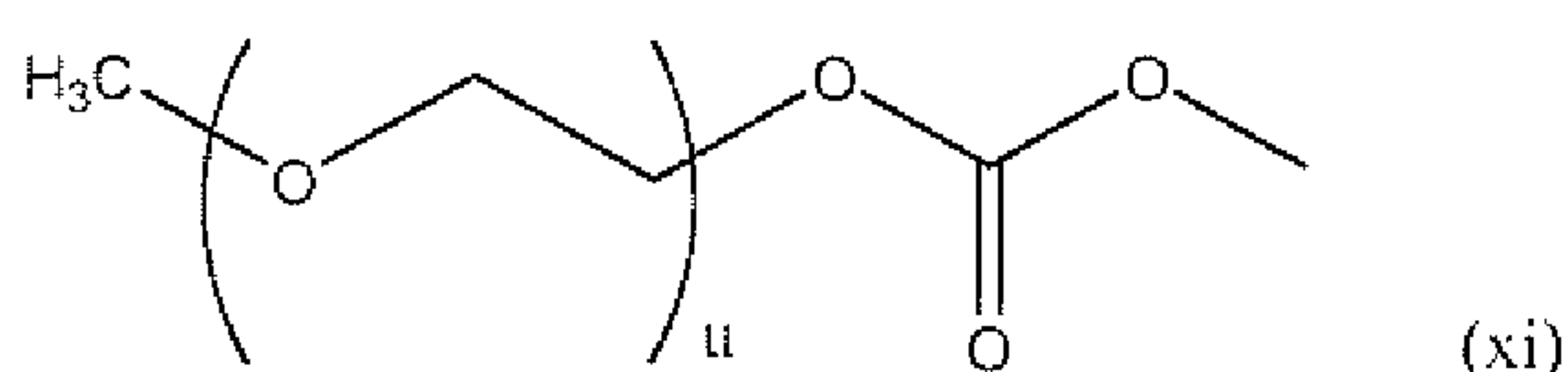
(iii)







and



5

wherein:

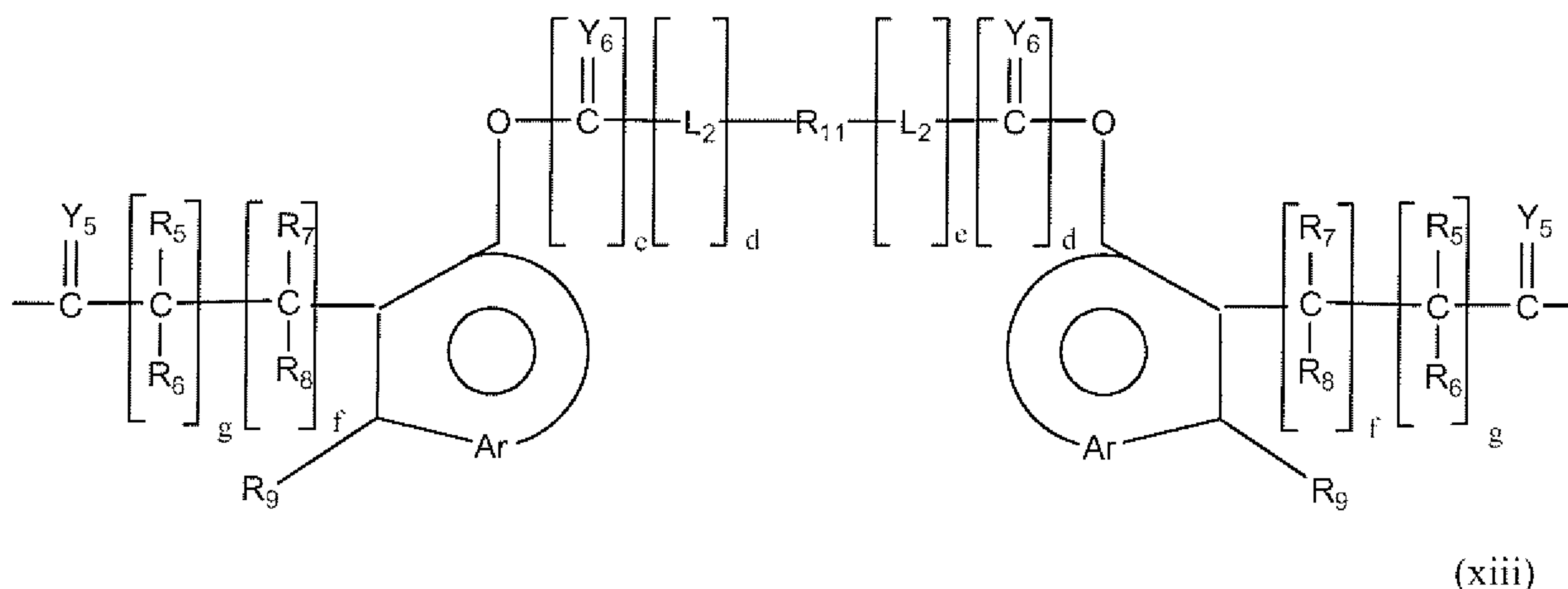
$R_{10-11}$ , and  $R_{22-23}$  may be the same or different and are independently selected non-antigenic polymer residues;

$R_{3-9}$ ,  $R_{12-21}$  and  $R_{24}$  (see below) are the same or different and are each independently selected from among hydrogen,  $C_{1-6}$  alkyls,  $C_{3-12}$  branched alkyls,  $C_{3-8}$  cycloalkyls,  $C_{1-6}$  substituted alkyls,  $C_{3-8}$  substituted cycloalkyls, aryls, substituted aryls, aralkyls,  $C_{1-6}$  heteroalkyls, substituted  $C_{1-6}$  heteroalkyls,  $C_{1-6}$  alkoxy, phenoxy and  $C_{1-6}$  heteroalkoxys;

10





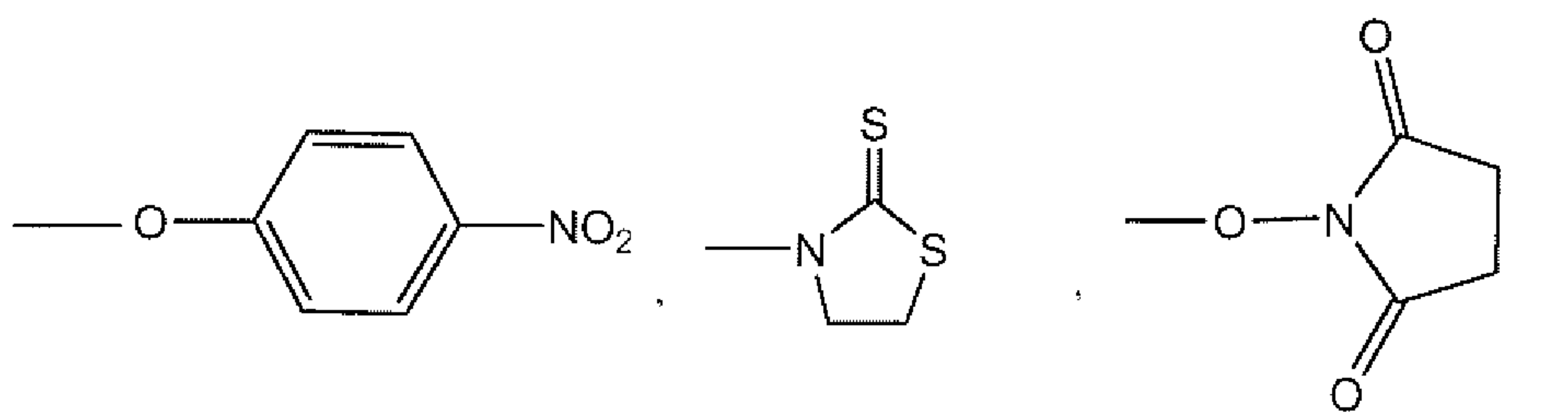


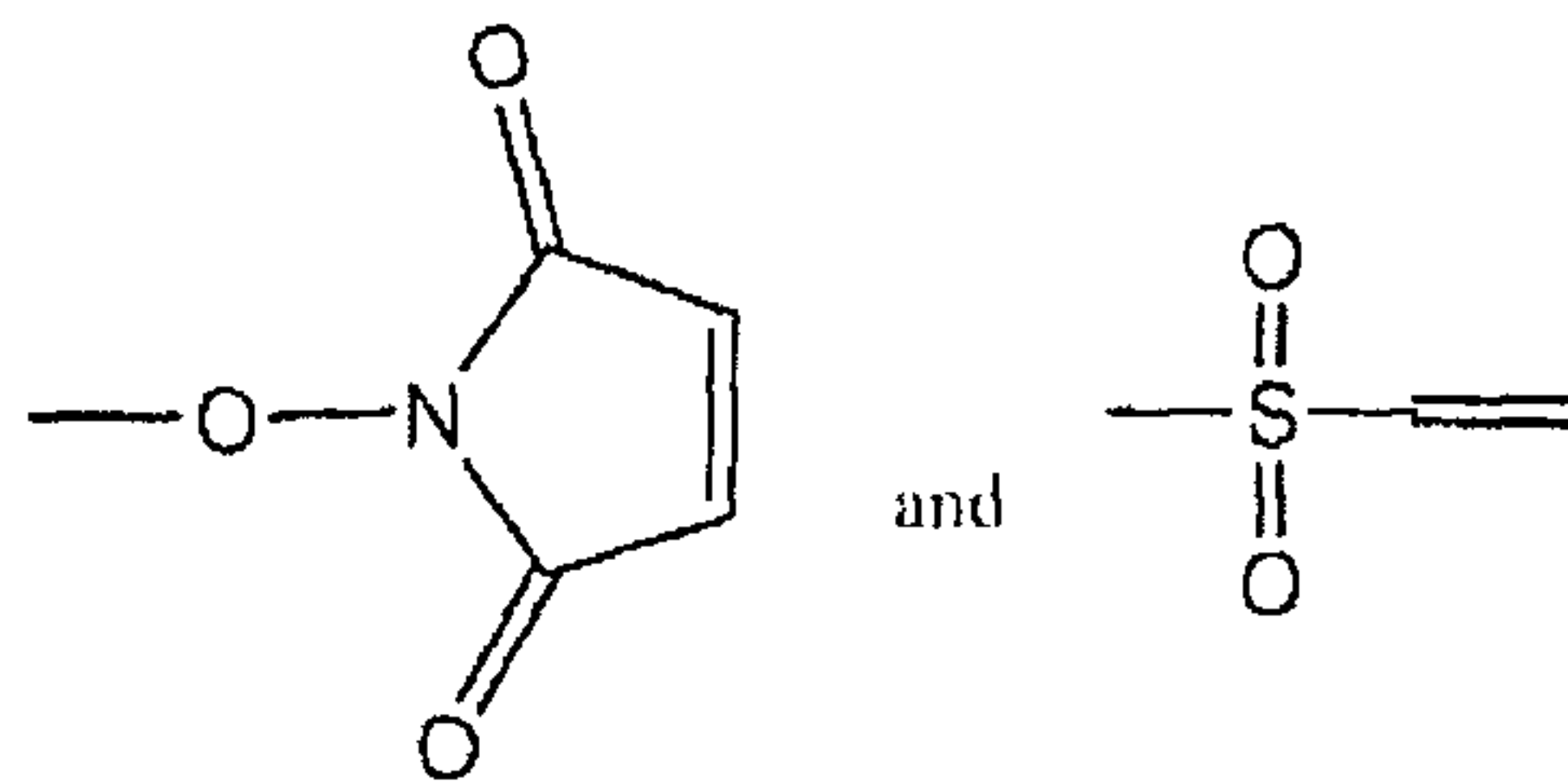
wherein all variables are the same as that set forth above.

Alternatively and/or preferably, multiple PEG strands are attached to the ADA. In these aspects, the ADA polymer conjugates can include at least 5 polyethylene glycol strands up to 80 strands attached to epsilon amino groups of Lys on the enzyme, but preferably, can include about 11-18 PEG strands attached to epsilon amino groups of Lys on the enzyme.

While the ADA is conjugated to from about 11 to about 18 PEG molecules per enzyme molecule, via lysine linkages, the ratio of PEG to ADA can be varied in order to modify the physical and kinetic properties of the combined conjugate to fit any particular clinical situation.

The activated polymers which can be employed to make the ADA conjugates will naturally correspond directly with the polymer portions described above. The chief difference is the presence of a leaving or activating group, sometimes designated herein as B<sub>1</sub>, which facilitates the attachment of the polymer system to an amine group (e.g., epsilon amine group of lysine) found on the ADA. Thus, compounds (i) - (xiii) include a leaving or activating group such as:





or other suitable leaving or activating groups such as N-hydroxybenzotriazolyl, halogen, N-hydroxyphthalimidyl, imidazolyl, O-acyl ureas, pentafluorophenol or 2,4,6-trichlorophenol or other suitable leaving groups apparent to those of ordinary skill, found in the place where the ADA attaches after the conjugation reaction.

Some preferred activated PEGs include those disclosed in commonly assigned U.S. Patent Nos. 5,122,614, 5,324,844, 5,612,460 and 5,808,096 (succinimidyl carbonate-activated polyethylene glycol (SC-PEG) and related activated PEG's), and U.S. Patent No. 5,349,001 (cyclic imide thione activated PEG's).

As will be appreciated by those of ordinary skill such conjugation reactions typically are carried out in a suitable buffer using a several-fold molar excess of activated PEG. Some preferred conjugates made with linear PEGs like the above mentioned SC-PEG can contain, on average, from about 1 to about 80 PEG strands per enzyme. Consequently, for these, molar excesses of several hundred fold, e.g., 200-1000x can be employed. The molar excess used for branched polymers and polymers attached to the enzyme will be lower and can be determined using the techniques described in the patents and patent applications describing the same that are mentioned herein below.

For purposes of the present invention, activated groups are to be understood as those groups which are capable of reacting with an amine group (nucleophile) found on an ADA, e.g. on a Lys.

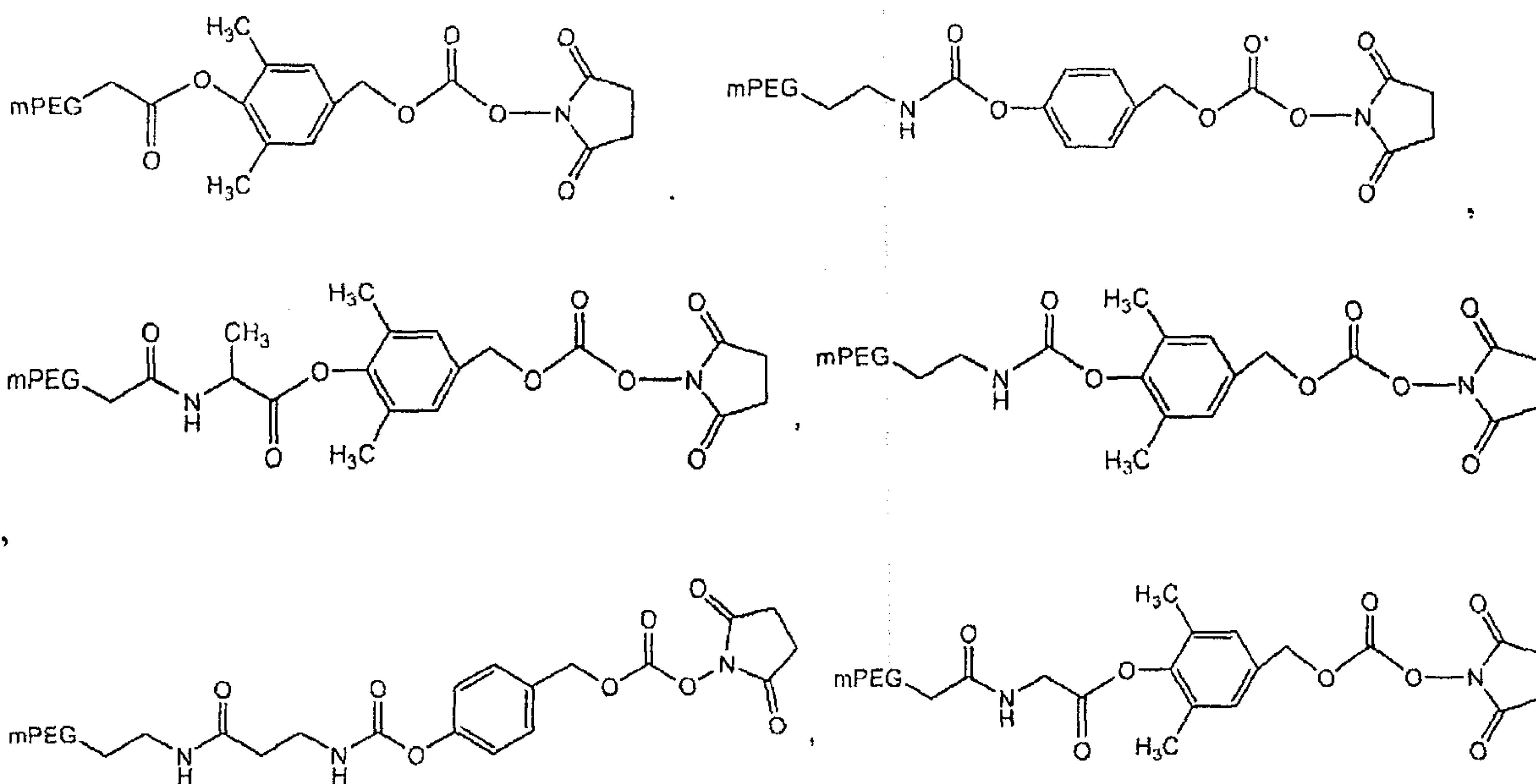
For purposes of the present invention, the foregoing are also referred to as activated polymer linkers. The polymer residues are preferably polyalkylene oxide-based and more preferably polyethylene glycol (PEG) based wherein the PEG is linear, branched or multi-armed.

Referring now to the polymers described above, it can be seen that the Ar is a moiety which forms a multi-substituted aromatic hydrocarbon or a multi-substituted heterocyclic group. A key feature is that the Ar moiety is aromatic in nature. Generally, to be aromatic,

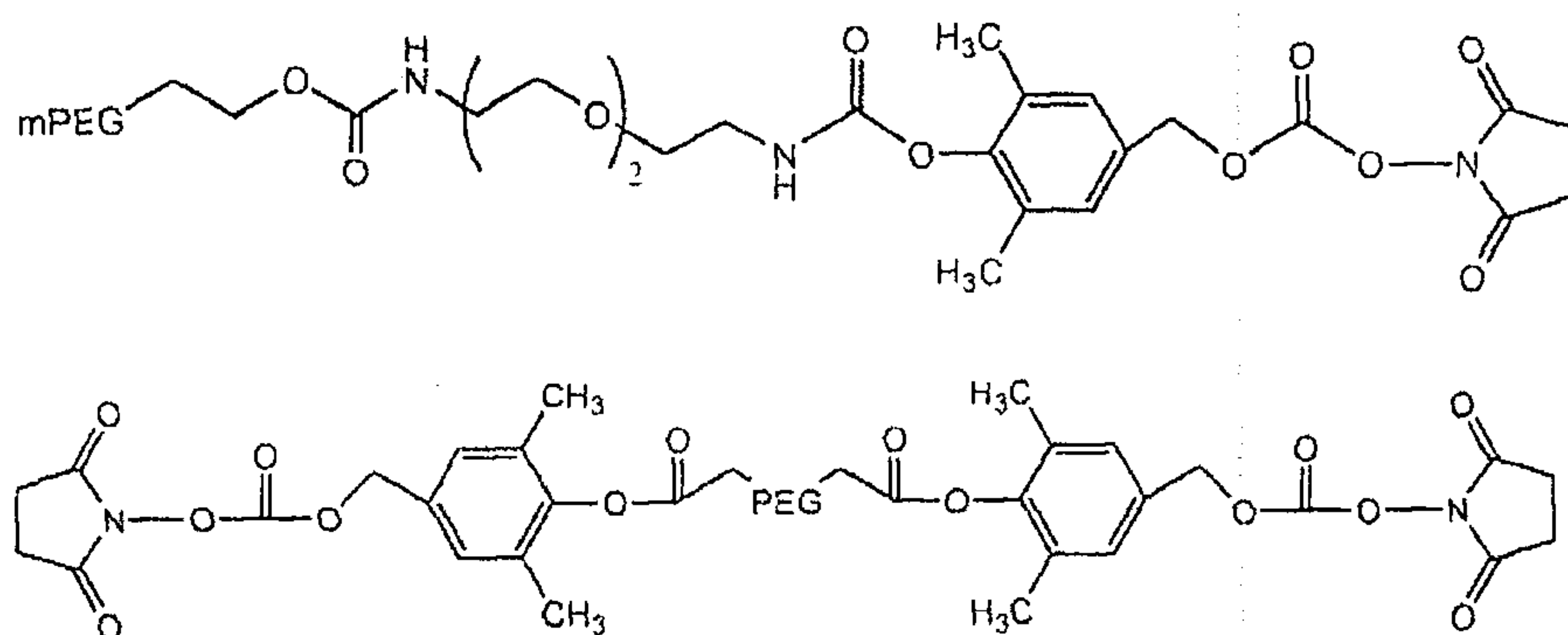
the pi electrons must be shared within a "cloud" both above and below the plane of a cyclic molecule. Furthermore, the number of pi electrons must satisfy the Huckle rule ( $4n+2$ ). Those of ordinary skill will realize that a myriad of moieties will satisfy the aromatic requirement of the moiety and thus are suitable for use herein.

5 In some preferred aspects of the invention, the activated polymer linkers of the polymeric systems based on benzyl elimination or trimethyl lock lactonization are prepared in accordance with commonly-assigned U.S. Patent Nos. 6,180,095, 6,720,306, 5,965,119, 6,624,142 and 6,303,569. Within this context, the following activated polymer linkers are preferred:

10



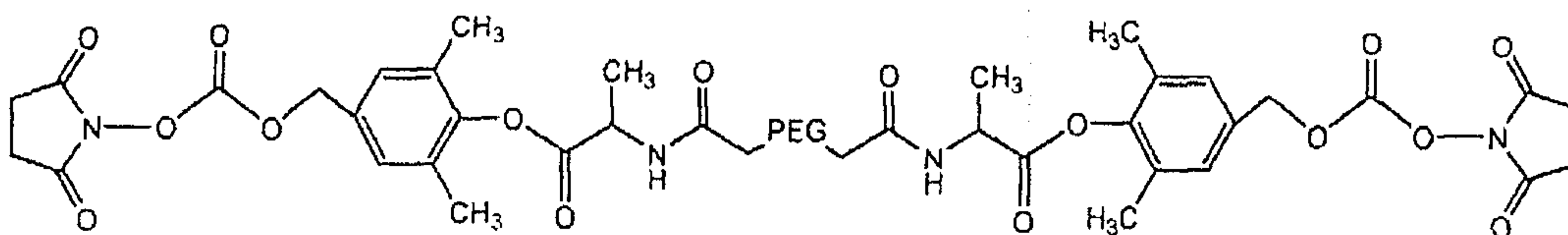
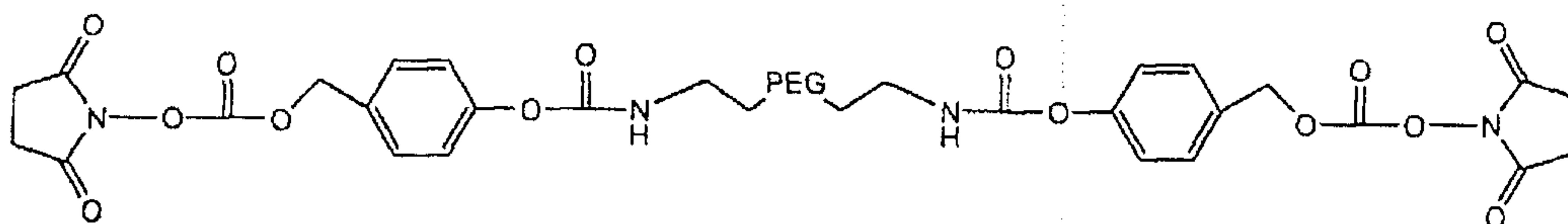
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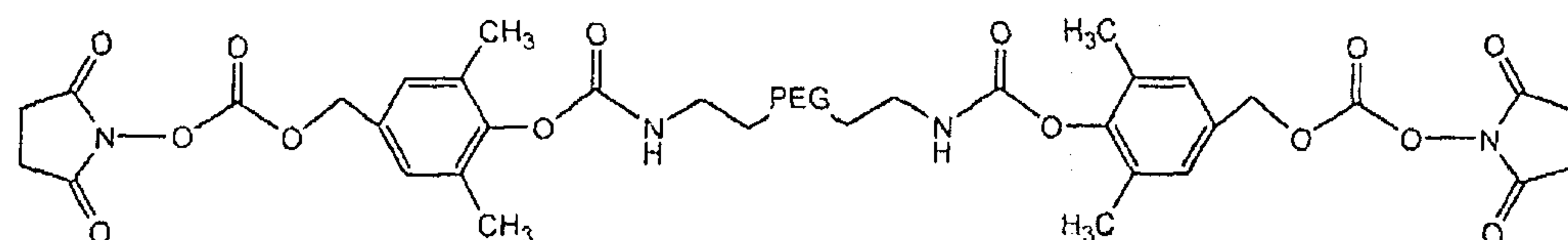


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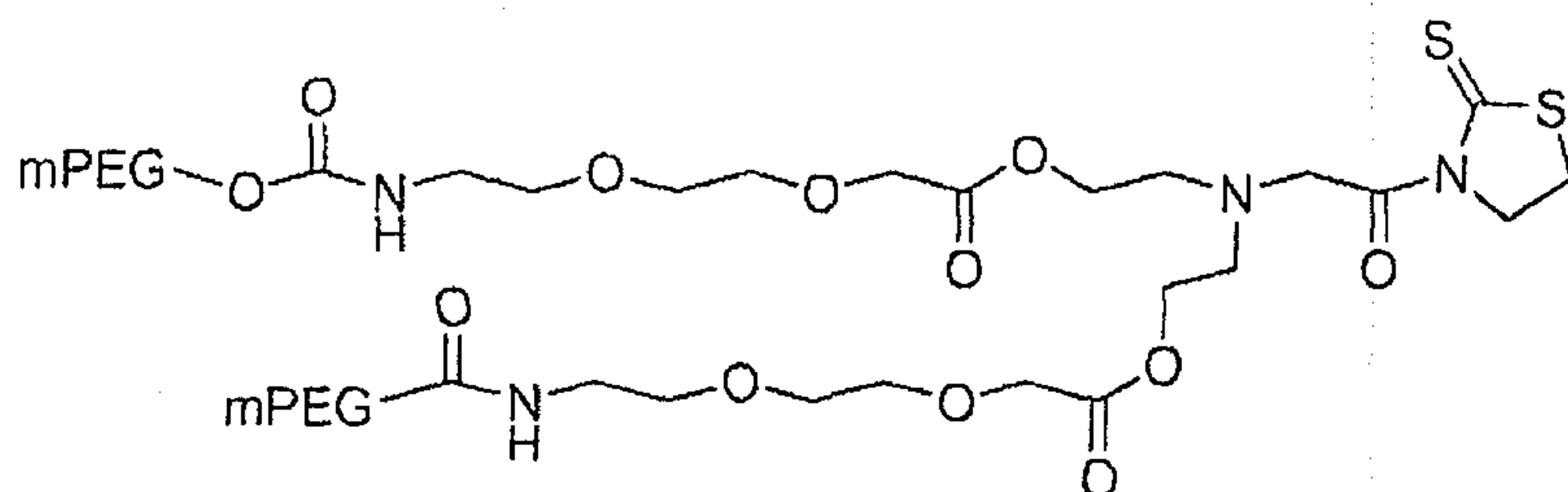


and



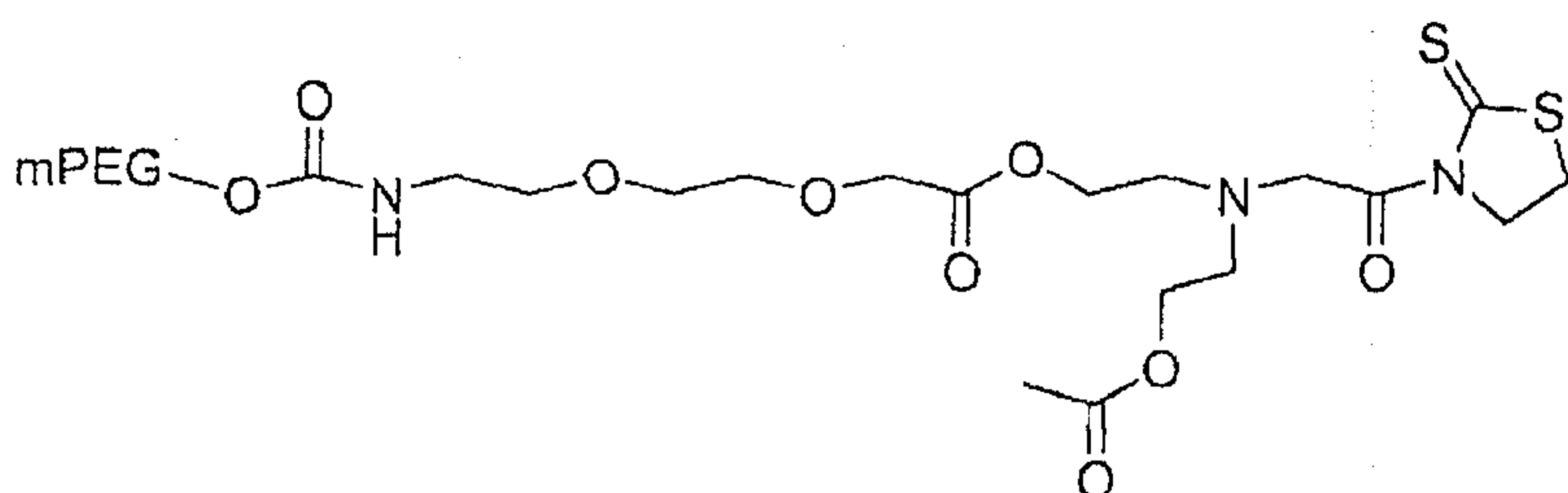
- 5 In one alternative aspect of the invention, the ADA polymer conjugates are made using certain bicine polymer residues such as those described in commonly assigned U.S. Patent Nos. 7,122,189 and 7,087,229 and U.S. Patent Publication No. 2007-0166276A1 and U.S. Patent Nos. 7,897,647 and 7,413,738.

A few of the preferred activated polymers include:



10

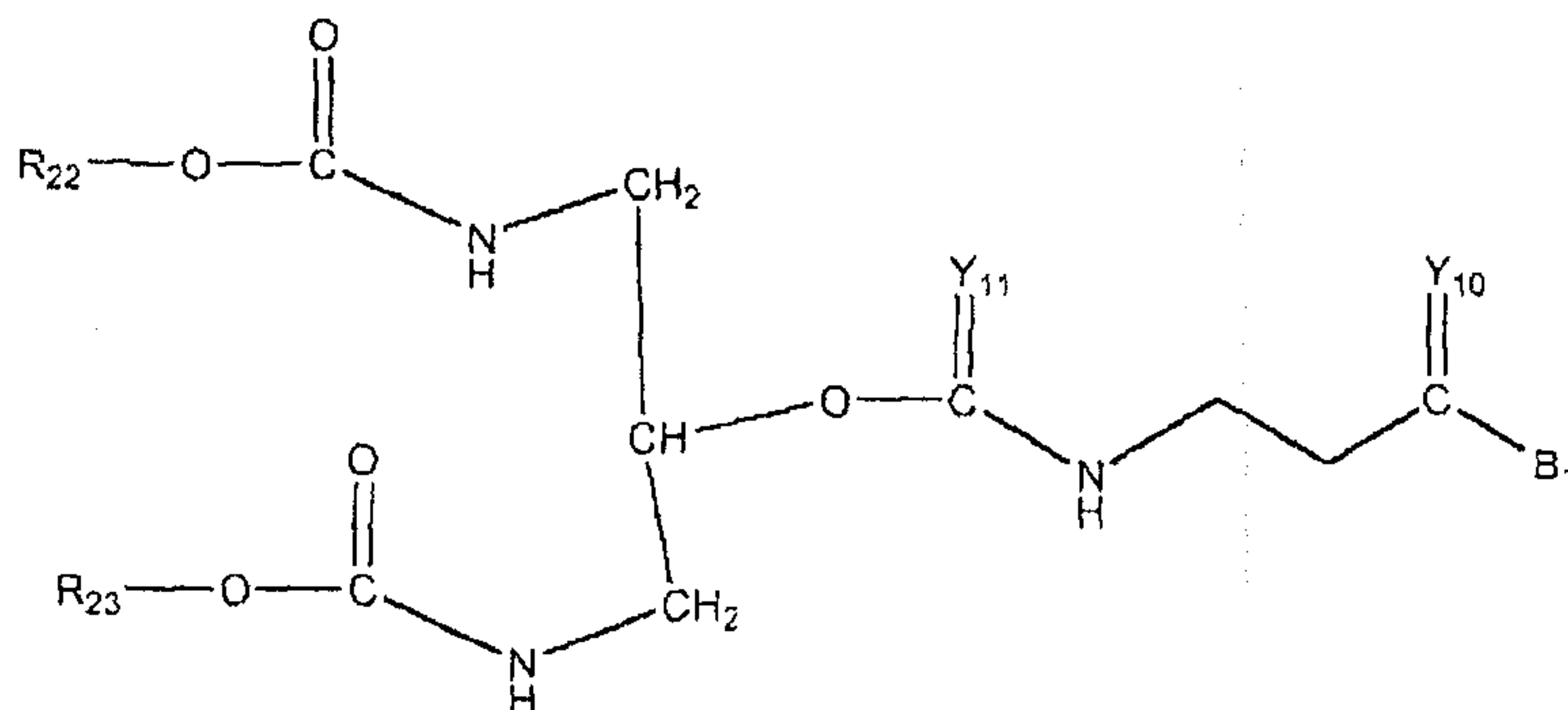
and



It should also be understood that the leaving or activating group shown above is only one of the suitable groups and the others mentioned herein can also be used without undue experimentation.

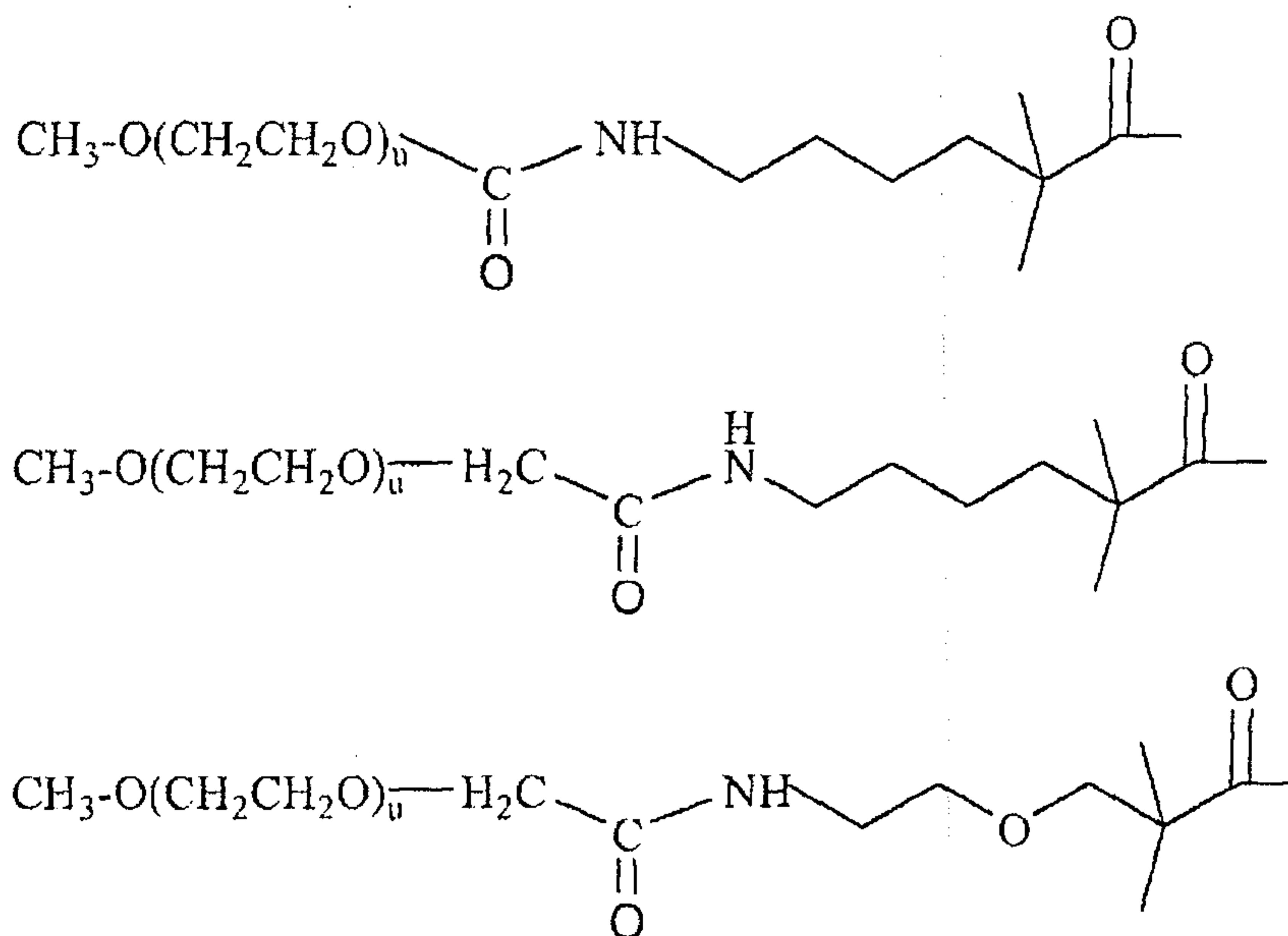
In alternative aspects, the activated polymer linkers are prepared using branched polymer residues such as those described commonly assigned U.S. Patent Nos. 5,681,567, 5,756,593, 5,643,575; 5,919,455, 6,113,906, 6,153,655, 6,395,266 and 6,638,499, 6,251,382 and 6,824,766. Such activated

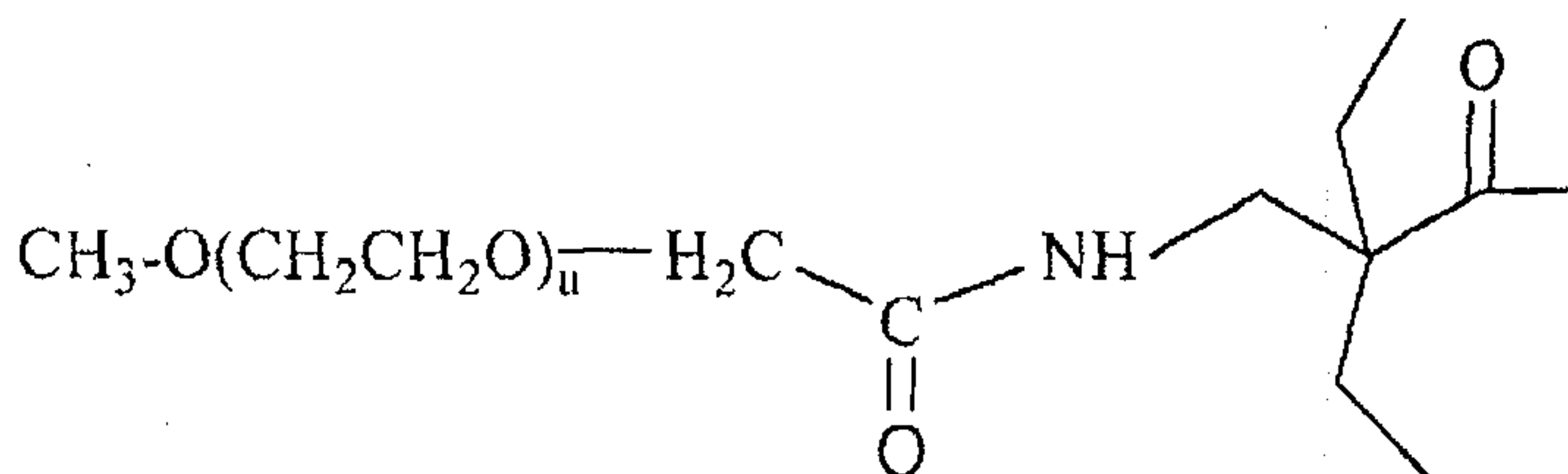
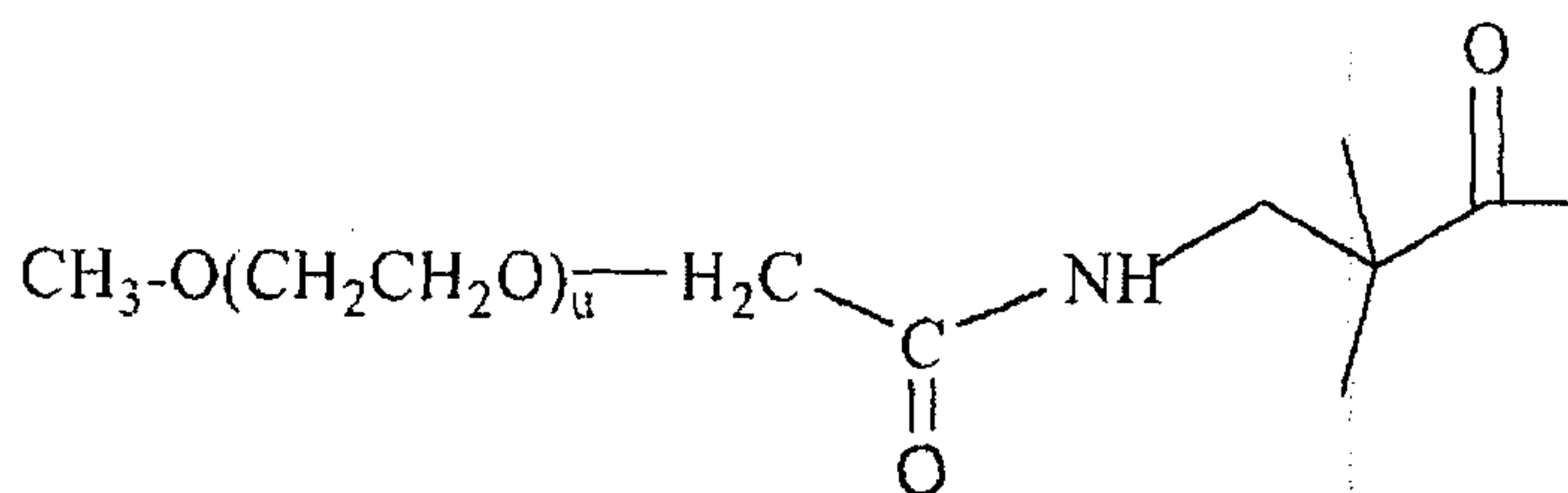
5 polymers correspond to polymer systems (iv) - (ix) with the following being representative:



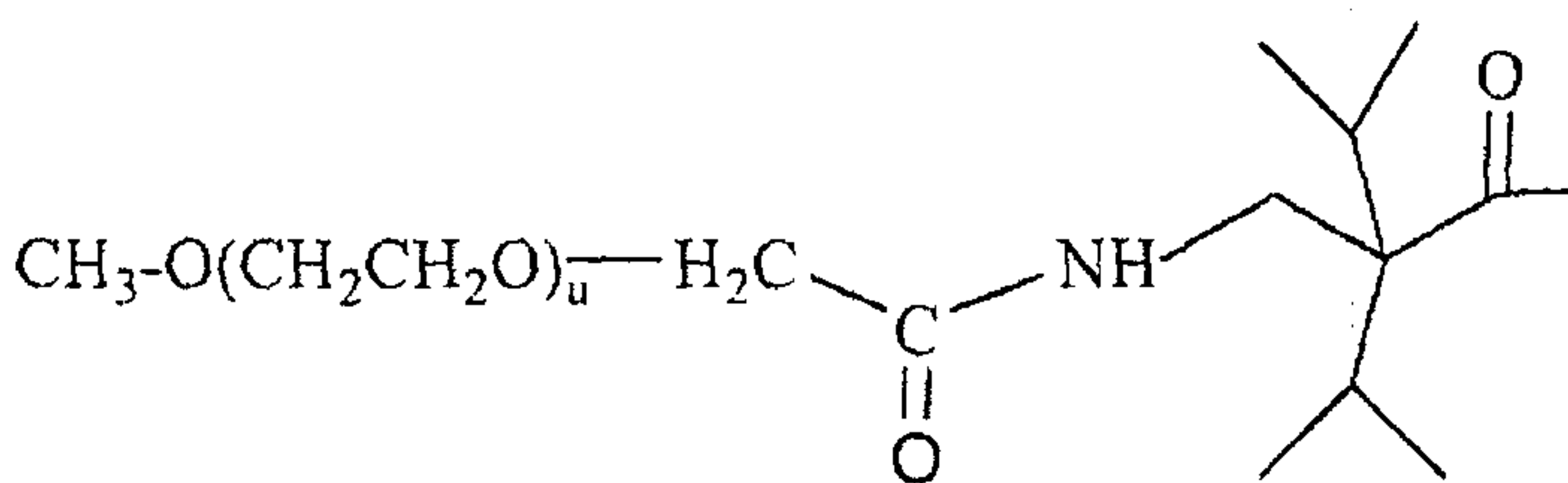
wherein  $B_1$  is an activating group and all variables are as previously defined.

In yet alternative aspects, the activated polymers can employ a hindered ester-based linker. See PCT/US07/78593 entitled "Polyalkylene Oxides Having Hindered Ester-Based  
10 Biodegradable Linkers". For example, a non-limiting list of such compounds includes:





and



5 wherein (u) is an integer ranging from about 10 to about 2300, to preferably provide polymers having a total molecular weight of from about 4,000 to about 45,000.

In one preferred embodiment, the activated polyethylene glycol is one which provides a urethane linkage or amide-linkage with the protein.

10 Methods of preparing polymers having terminal carboxylic acids in high purity are described in U.S. Patent No. 7,989,554.

The methods include first preparing a tertiary alkyl ester of a polyalkylene oxide followed by conversion to the carboxylic acid derivative thereof. The first step of the preparation of the PAO carboxylic acids of the process includes forming an intermediate such as *t*-butyl ester of polyalkylene oxide carboxylic acid. This intermediate is  
 15 formed by reacting a PAO with a *t*-butyl haloacetate in the presence of a base such as potassium *t*-butoxide. Once the *t*-butyl ester intermediate has been formed, the carboxylic acid derivative of the polyalkylene oxide can be readily provided in purities exceeding 92%, preferably exceeding 97%, more preferably exceeding 99% and most preferably exceeding 99.5% purity.

20 In yet alternative aspects, polymers having terminal amine groups can be employed to make the ADA conjugates. The methods of preparing polymers containing terminal amines



in high purity are described in US Patent Nos. 7,868,131 and 7,569,657.

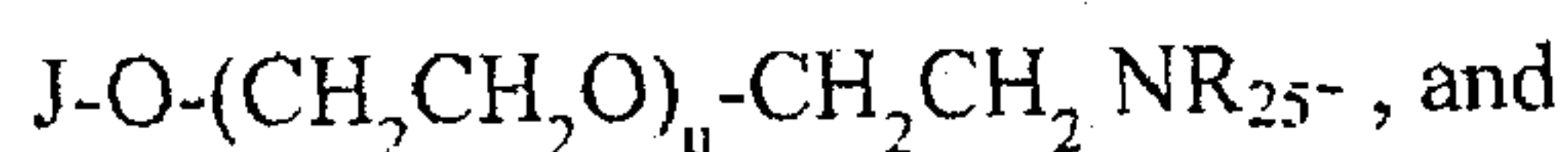
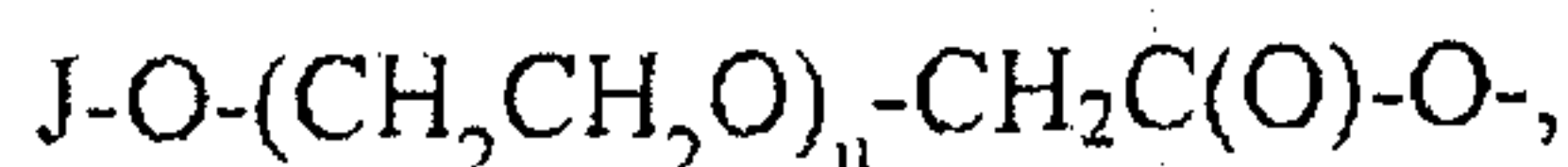
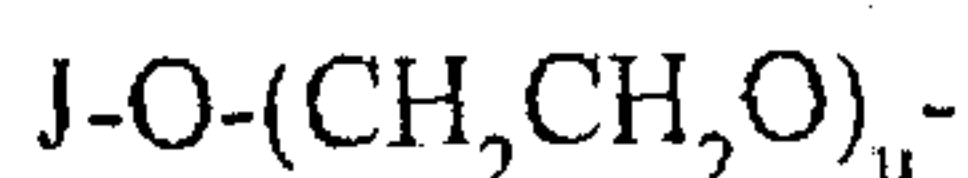
For example, polymers having azides react with phosphine-based reducing agent such as triphenylphosphine or an alkali metal borohydride reducing agent such as NaBH<sub>4</sub>. Alternatively, polymers including leaving groups react with protected amine salts such as potassium salt of methyl-tert-butyl imidodicarbonate (KNMeBoc) or the potassium salt of di-tert-butyl imidodicarbonate (KNBoc<sub>2</sub>) followed by deprotecting the protected amine group. The purity of the polymers containing the terminal amines formed by these processes is greater than about 95% and preferably greater than 99%.

10

### 1. Substantially Non-Antigenic Polymers

As stated above, R<sub>10-11</sub>, and R<sub>22-23</sub> are preferably each water soluble polymer residues which are preferably substantially non-antigenic such as polyalkylene oxides (PAO's) and more preferably polyethylene glycols such as mPEG. For purposes of illustration and not limitation, the polyethylene glycol (PEG) residue portion of R<sub>10-11</sub>, and R<sub>22-23</sub> can be selected from among:

15



20



wherein:

(u) is the degree of polymerization, i.e. from about 10 to about 2,300;

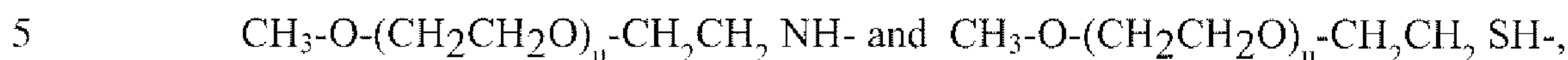
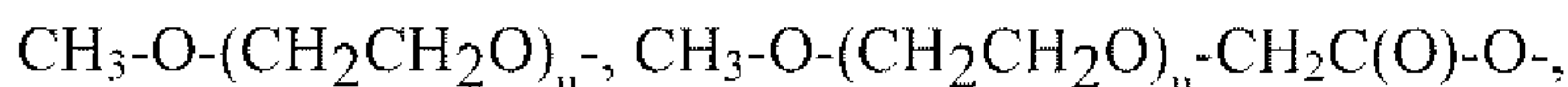
R<sub>25</sub> is selected from among hydrogen, C<sub>1-6</sub> alkyls, C<sub>2-6</sub> alkenyls, C<sub>2-6</sub> alkynyls, C<sub>3-12</sub> branched alkyls, C<sub>3-8</sub> cycloalkyls, C<sub>1-6</sub> substituted alkyls, C<sub>2-6</sub> substituted alkenyls, C<sub>2-6</sub> substituted alkynyls, C<sub>3-8</sub> substituted cycloalkyls, aryls substituted aryls, aralkyls, C<sub>1-6</sub> heteroalkyls, substituted C<sub>1-6</sub> heteroalkyls, C<sub>1-6</sub> alkoxy, phenoxy and C<sub>1-6</sub> heteroalkoxy, and

25

J is a capping group, i.e., a group which is found on the terminal of the polymer and, in some aspects, can be selected from any of NH<sub>2</sub> (or CH<sub>2</sub>CH<sub>2</sub>NH<sub>2</sub>), H, SH (or CH<sub>2</sub>CH<sub>2</sub>SH),

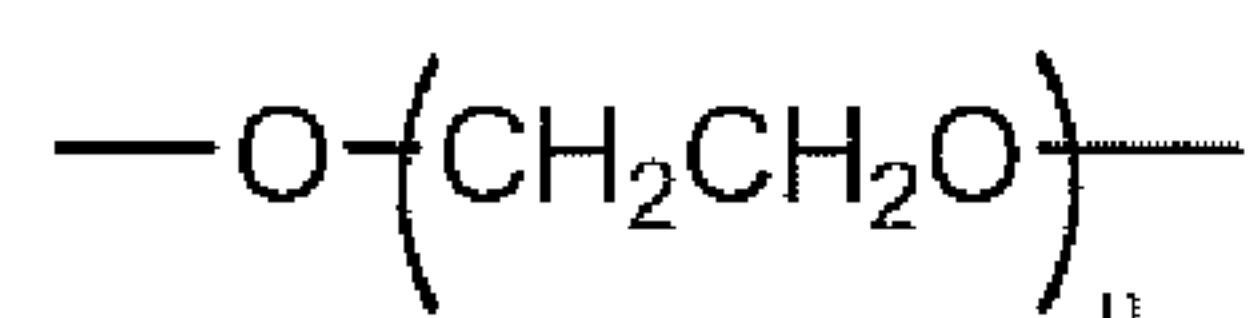
CO<sub>2</sub>H (or CH<sub>2</sub>CO<sub>2</sub>H), C<sub>1-6</sub> alkyls, preferably methyl, or other PEG terminal activating groups, as such groups are understood by those of ordinary skill.

In one particularly preferred embodiment, R<sub>10-11</sub>, and R<sub>22-23</sub> are selected from among,



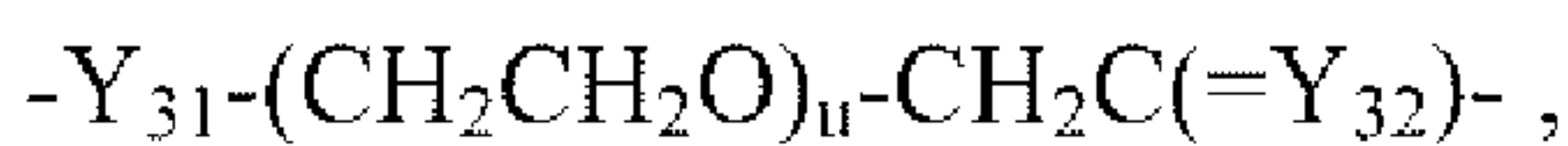
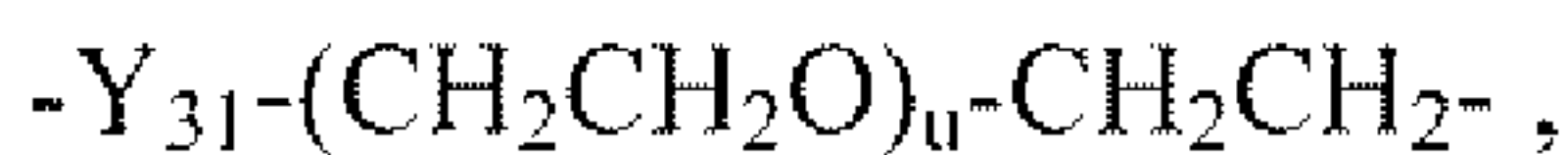
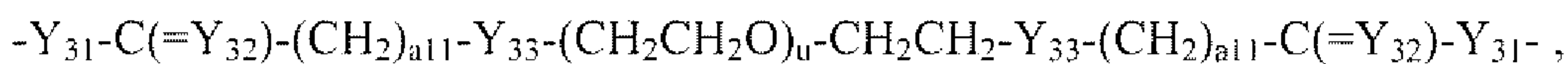
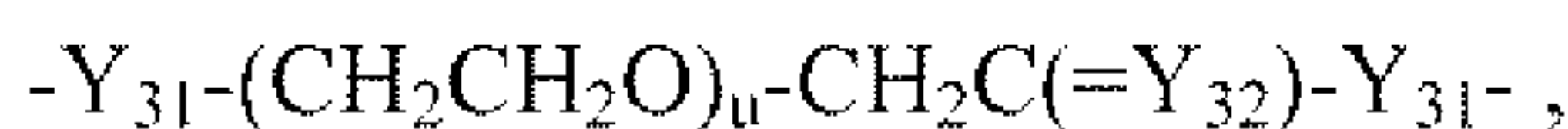
where (u) is a positive integer, preferably selected so that the average total molecular weight of the polymer portion ranges from about 2,000 to about 100,000 Da. More preferably, R<sub>10-11</sub>, and R<sub>22-23</sub> independently have an average total molecular weight of from about 4,000 Da to about 4,5000 Da, with a weight average molecular weight of from about 5,000 Da being most preferred. Other molecular weights are also contemplated so as to accommodate the needs of the artisan.

PEG is generally represented by the structure:



and R<sub>10-11</sub>, and R<sub>22-23</sub> preferably comprise residues of this formula. The degree of polymerization for the polymer represents the number of repeating units in the polymer chain and is dependent on the molecular weight of the polymer.

Alternatively, the polyethylene glycol (PEG) residue portion of the invention can be represented by the structure:



wherein:

Y<sub>31</sub> and Y<sub>33</sub> are independently O, S, SO, SO<sub>2</sub>, NR<sub>33</sub> or a bond;

Y<sub>32</sub> is O, S, or NR<sub>34</sub>;

30 R<sub>31-34</sub> are independently selected from among hydrogen, C<sub>1-6</sub> alkyl, C<sub>2-6</sub> alkenyl,

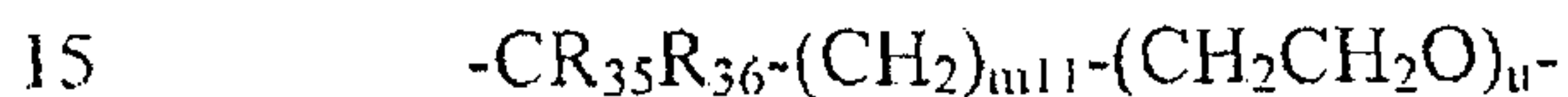
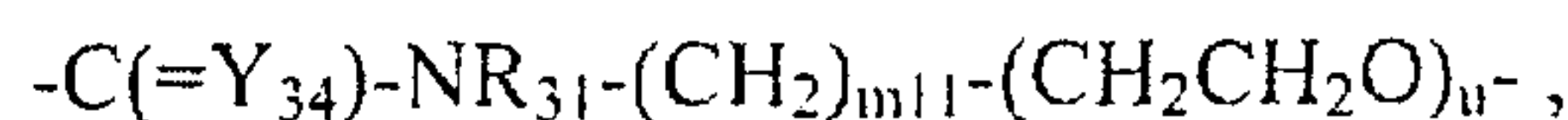
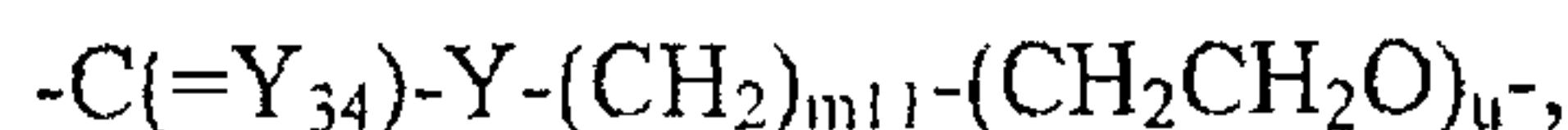


C<sub>2-6</sub> alkynyl, C<sub>3-19</sub> branched alkyl, C<sub>3-8</sub> cycloalkyl, C<sub>1-6</sub> substituted alkyl, C<sub>2-6</sub> substituted alkenyl, C<sub>2-6</sub> substituted alkynyl, C<sub>3-8</sub> substituted cycloalkyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, C<sub>1-6</sub> heteroalkyl, substituted C<sub>1-6</sub> heteroalkyl, C<sub>1-6</sub> alkoxy, aryloxy, C<sub>1-6</sub> heteroalkoxy, heteroaryloxy, C<sub>2-6</sub> alkanoyl, arylcarbonyl, C<sub>2-6</sub> alkoxy carbonyl, aryloxy carbonyl, C<sub>2-6</sub> alkanoyloxy, arylcarbonyloxy, C<sub>2-6</sub> substituted alkanoyl, substituted arylcarbonyl, C<sub>2-6</sub> substituted alkanoyloxy, substituted aryloxy carbonyl, C<sub>2-6</sub> substituted alkanoyloxy and substituted arylcarbonyloxy;

(a11), (a12), and (b11) are independently zero or a positive integer, preferably 0-6, and more preferably 0, 1, or 2; and

10 (u) is an integer from about 10 to about 2300.

As an example, the PEG can be functionalized in the following non-limiting manner:



wherein

R<sub>31</sub>, R<sub>35</sub> and R<sub>36</sub> are independently selected from among H, C<sub>1-6</sub> alkyls, aryls, substituted aryls, aralkyls, heteroalkyls, substituted heteroalkyls and substituted C<sub>1-6</sub> alkyls;

(m11) is zero or is a positive integer, and preferably 1 or 2;

20 Y<sub>34</sub> is O or S; and

(u) represents the degree of polymerization.

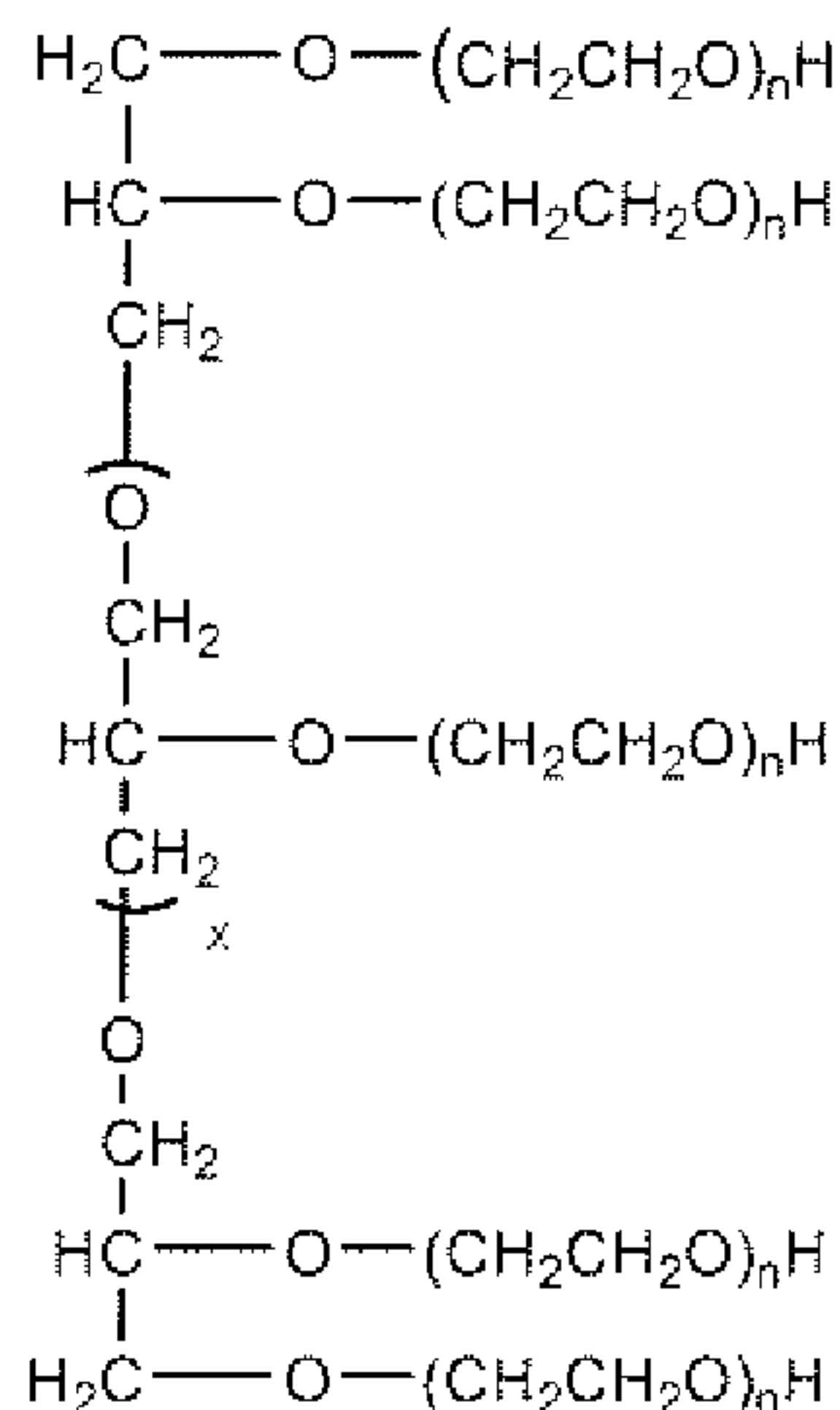
In these aspects, a capping group (J) such as methyl is attached to the terminal of the PEG.

For example, the conjugates of the present invention can be made by methods which include converting the multi-arm PEG-OH or "star-PEG" products such as those described in NOF Corp. Drug Delivery System catalog, Ver. 8, April 2006,

into a suitably activated polymer, using the activation techniques described in the aforementioned U.S. Patent Nos. 5,122,614 or 5,808,096. See also Shearwater Corporation's 2001 catalog "Polyethylene Glycol and Derivatives for Biomedical Application".



The multi-arm polymers contain four or more polymer arms and preferably four or eight polymer arms. For purposes of illustration and not limitation, the multi-arm polyethylene glycol (PEG) residue can be of the formula:

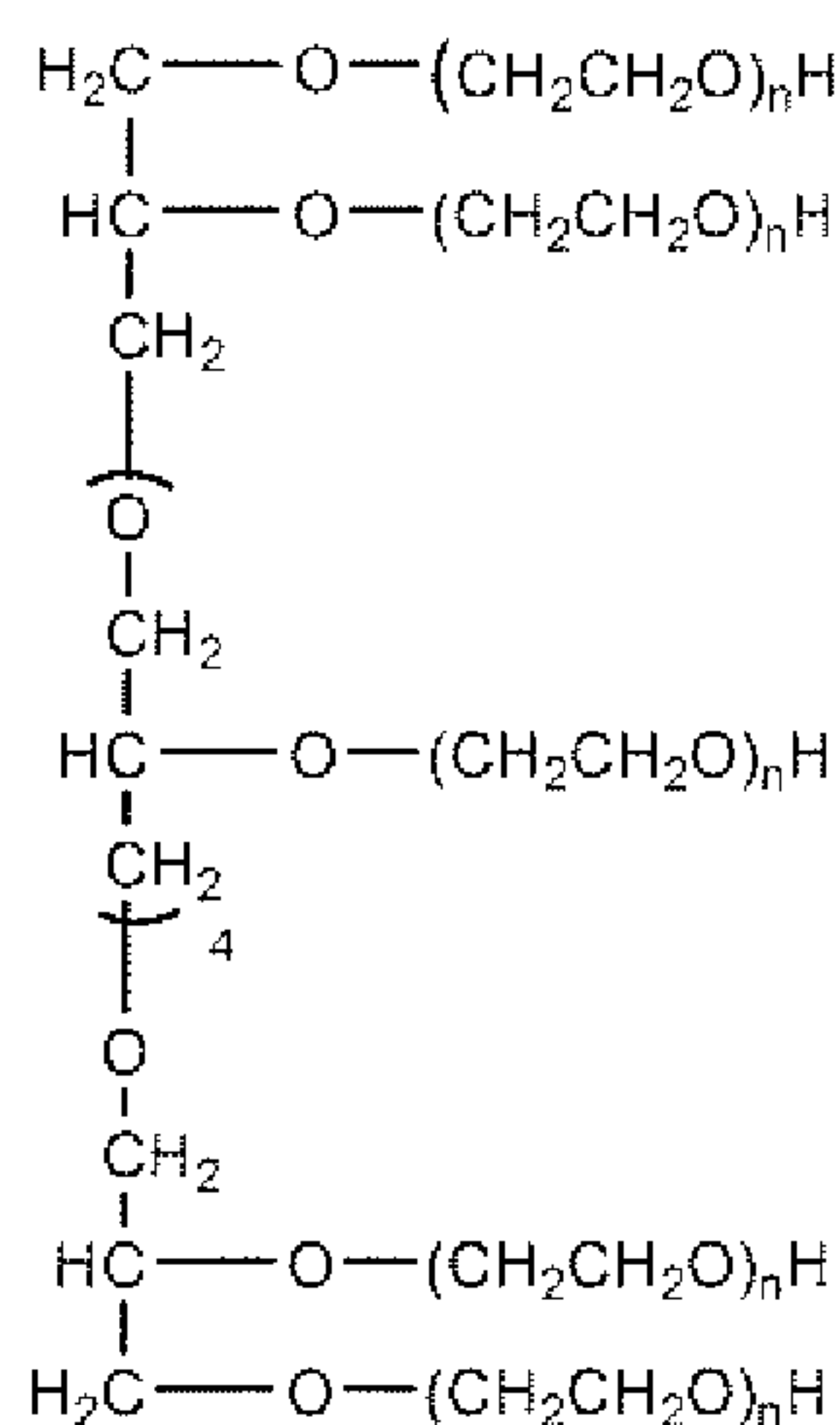


5 wherein:

(x) is 0 and a positive integer, i.e. from about 0 to about 28; and

(n) is the degree of polymerization.

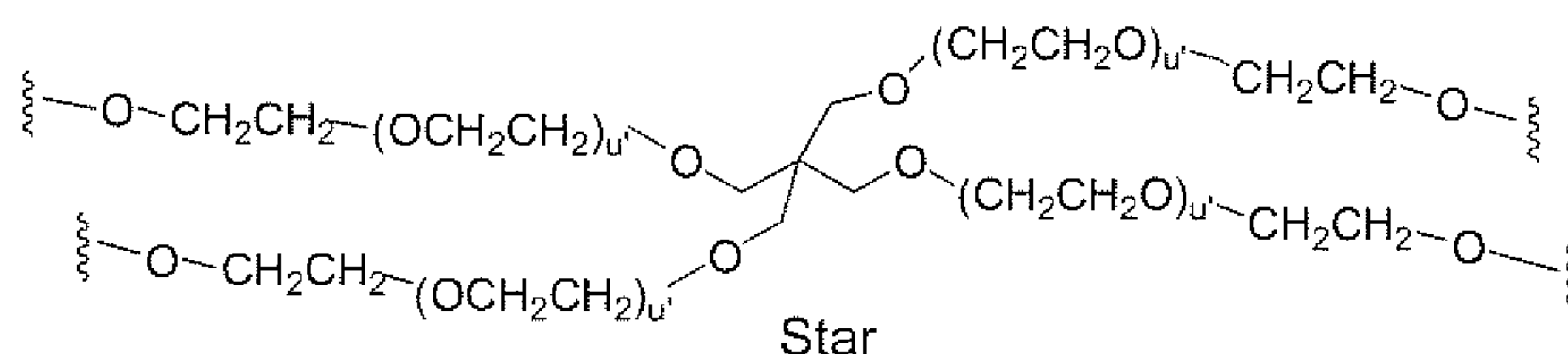
In one particular embodiment of the present invention, the multi-arm PEG has the structure:



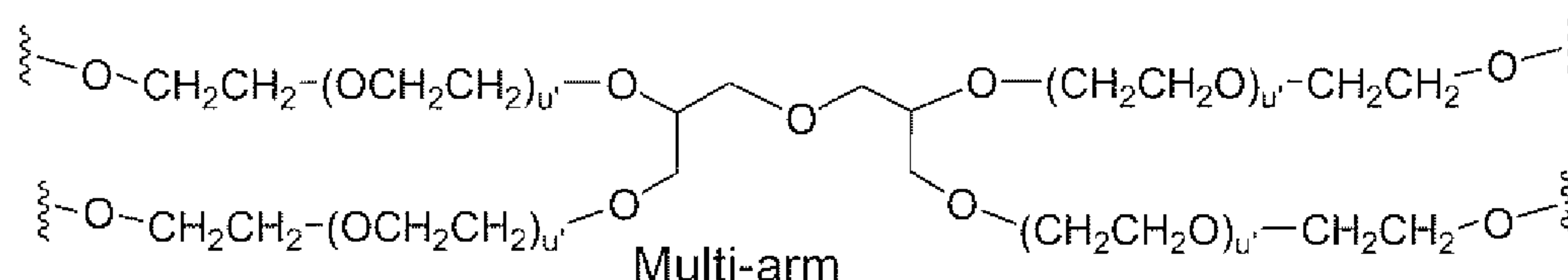
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wherein (n) is a positive integer. In one preferred embodiment of the invention, the polymers have a total molecular weight of from about 2,000 Da to about 100,000 Da, and preferably from 4,000 Da to 45,000 Da.

Specifically, the PEG can be of the formula:



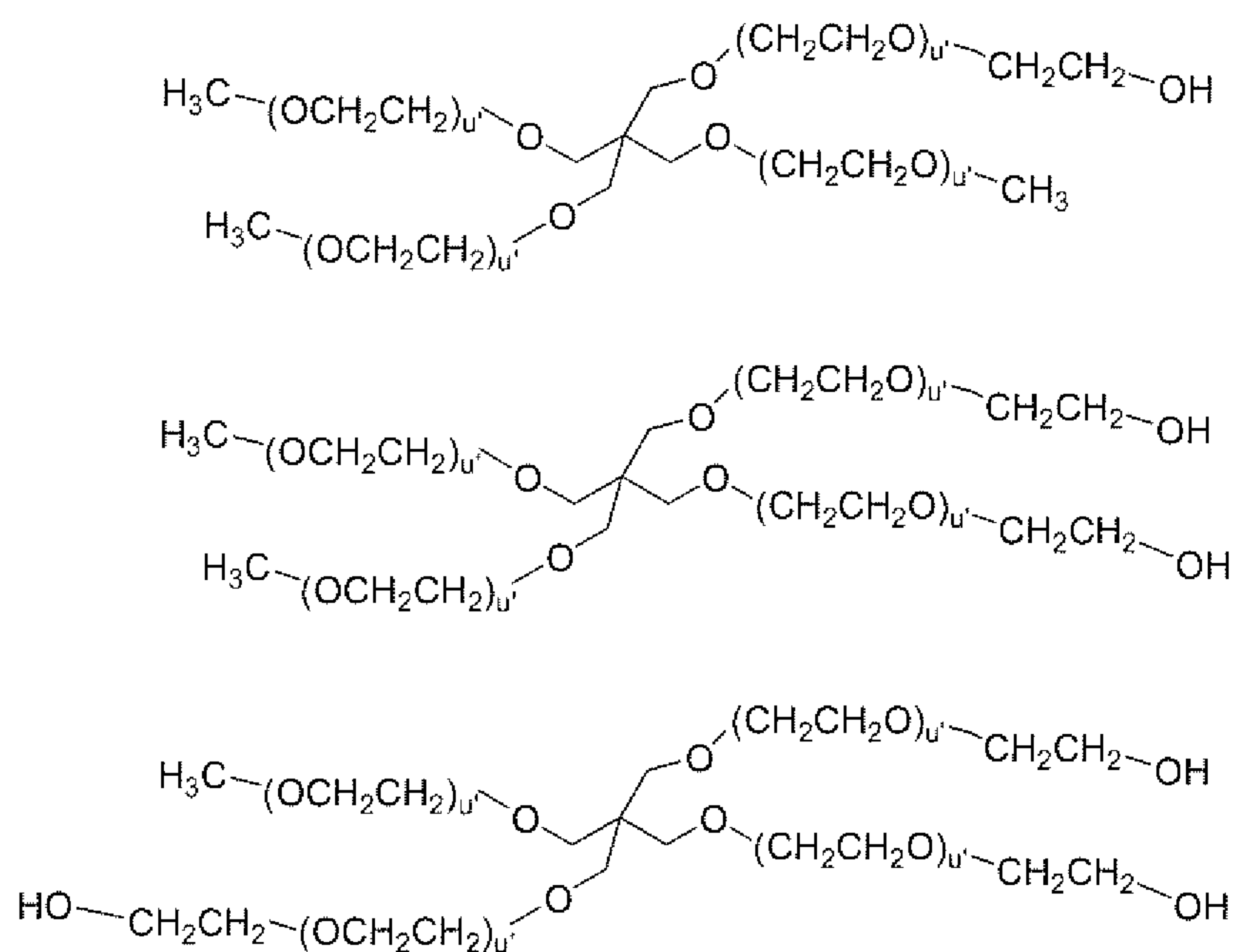
or



wherein:

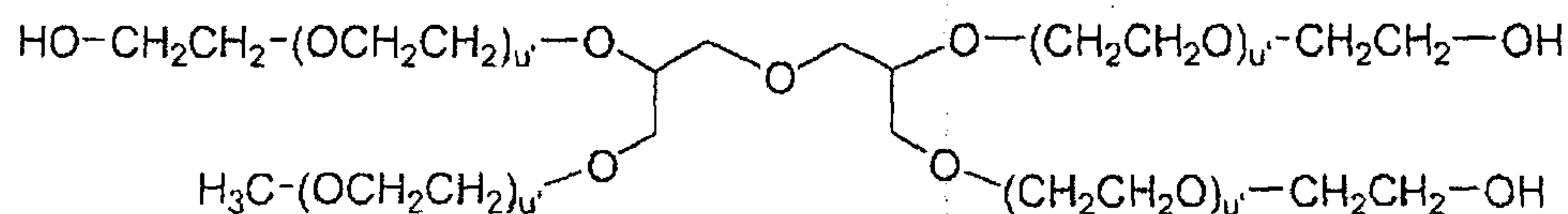
- 5 (u') is an integer from about 10 to about 570, to preferably provide polymers having a total molecular weight of from about 4,000 to about 45,000; and up to 3 terminal portions of the residue is/are capped with a methyl or other lower alkyl.

In some preferred embodiments, all 4 of the PEG arms are converted to suitable functional groups, i.e. SC, etc., for facilitating attachment to the recombinant protein. Such  
 10 compounds prior to conversion include:

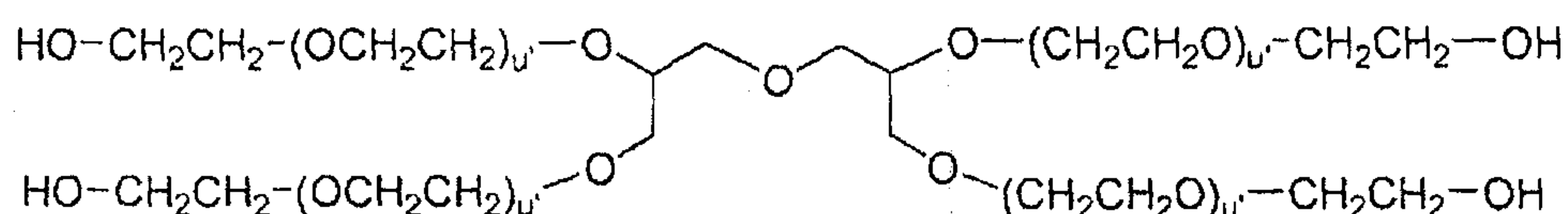








and



The polymeric substances included herein are preferably water-soluble at room  
 5 temperature. A non-limiting list of such polymers include polyalkylene oxide homopolymers  
 such as polyethylene glycol (PEG) or polypropylene glycols, polyoxyethylenated polyols,  
 copolymers thereof and block copolymers thereof, provided that the water solubility of the  
 block copolymers is maintained.

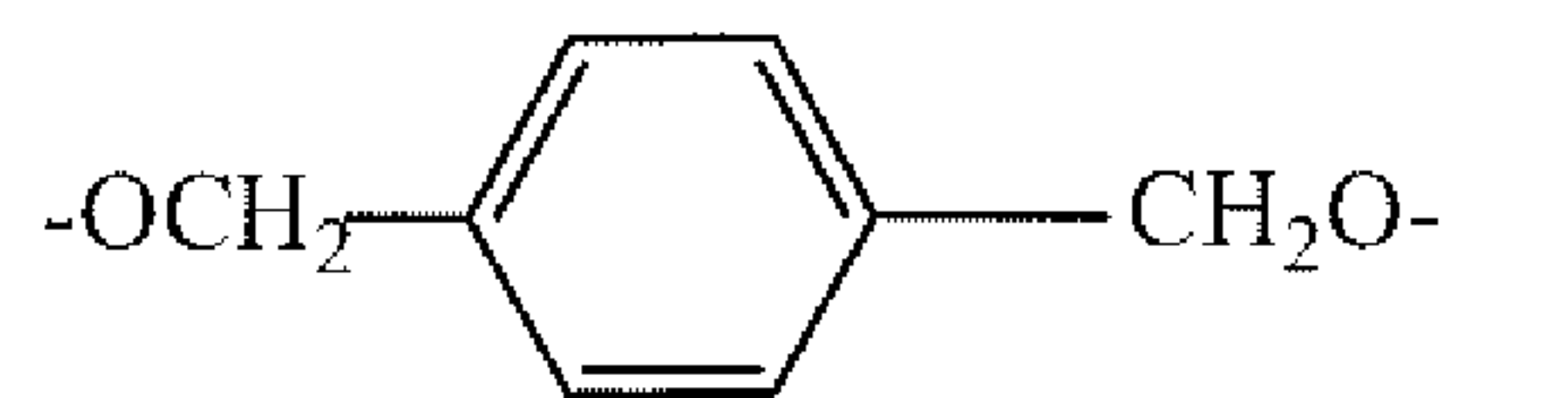
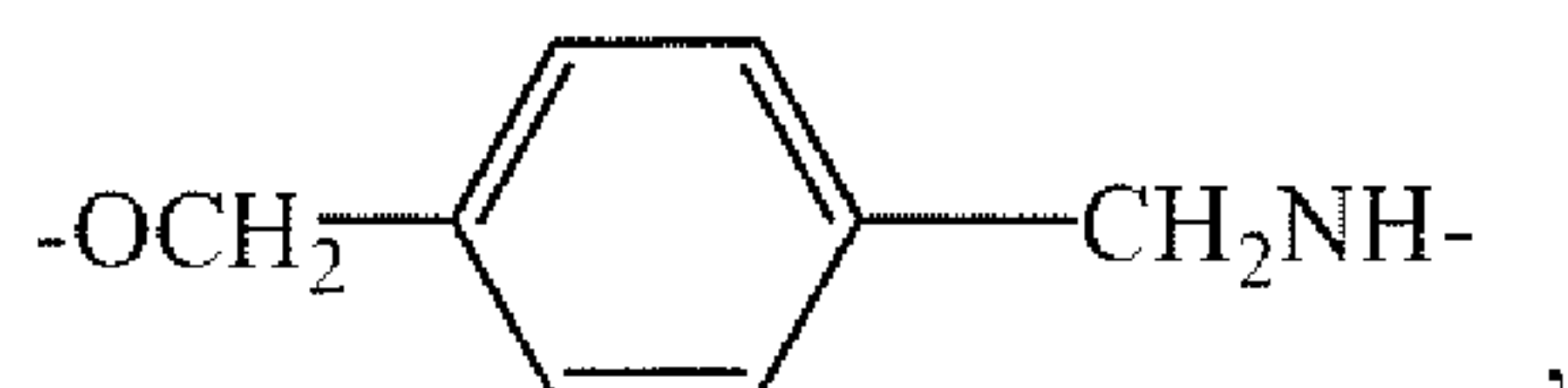
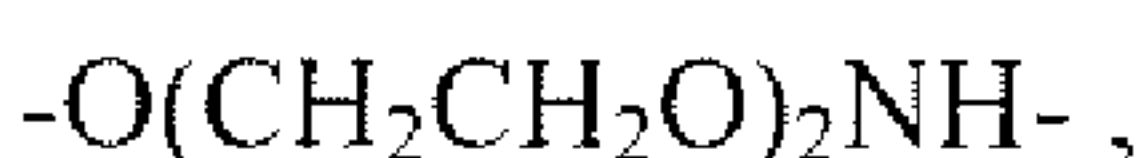
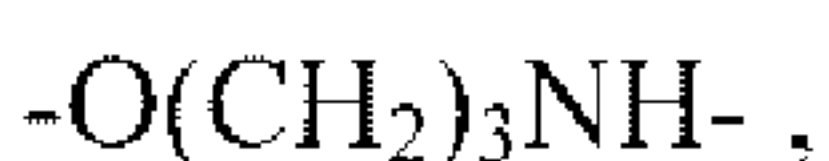
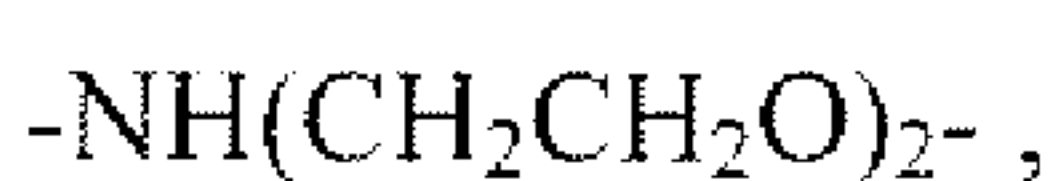
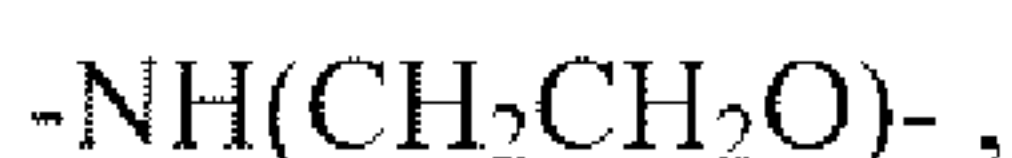
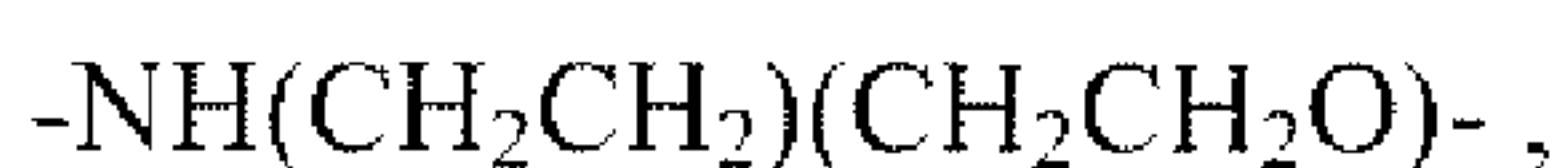
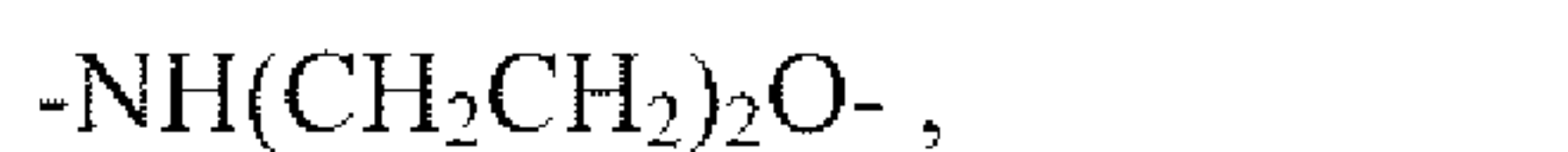
In a further embodiment, and as an alternative to PAO-based polymers, R<sub>10-11</sub>, and  
 10 R<sub>22-23</sub> are each optionally selected from among one or more effectively non-antigenic  
 materials such as dextran, polyvinyl alcohols, carbohydrate-based polymers,  
 hydroxypropylmeth-acrylamide (HPMA), polyalkylene oxides, and/or copolymers thereof.  
 See also commonly-assigned U.S. Patent No, 6,153,655.

It will be understood by those of ordinary skill that the  
 15 same type of activation is employed as described herein as for PAO's such as PEG. Those of  
 ordinary skill in the art will further realize that the foregoing list is merely illustrative and that  
 all polymeric materials having the qualities described herein are contemplated and that other  
 polyalkylene oxide derivatives such as the polypropylene glycols, etc. are also contemplated.

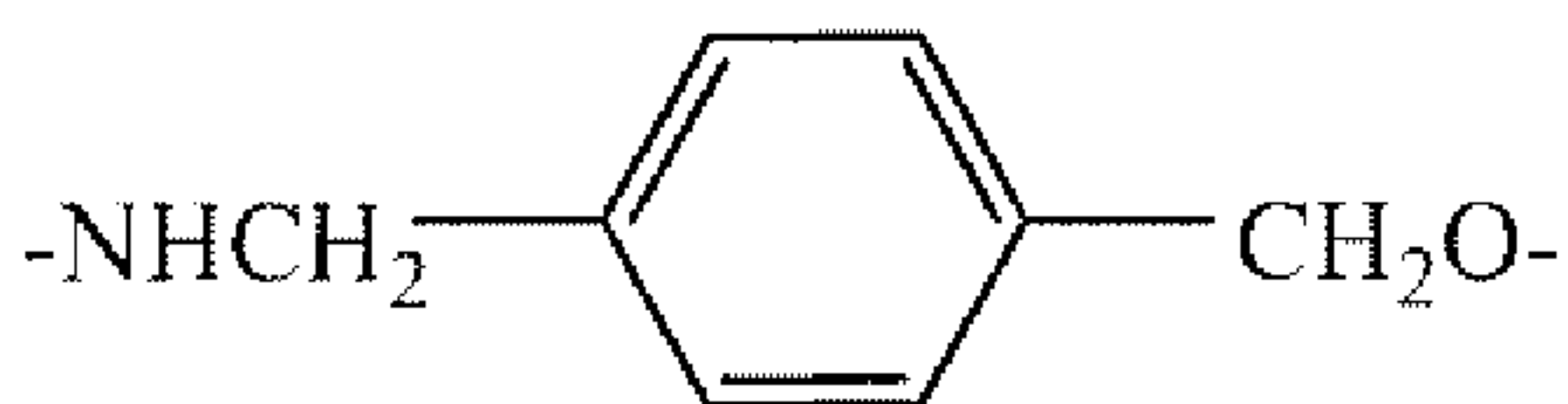
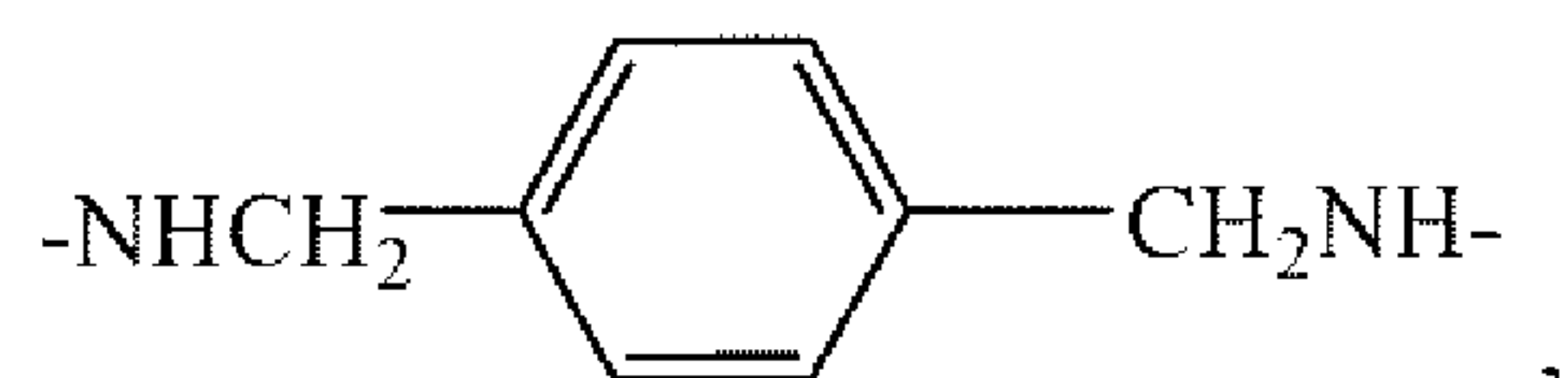
## 20 2. Bifunctional Linker Groups

In many aspects of the invention, L<sub>1-6</sub> and L<sub>8</sub> are linking groups which facilitate  
 attachment of the polymer strands, *e.g.* R<sub>10-11</sub>, and/or R<sub>22-23</sub>. The linkage provided can be  
 either direct or through further coupling groups known to those of ordinary skill. In this  
 aspect of the invention, L<sub>1-6</sub> and L<sub>8</sub> may be the same or different and can be selected from a  
 25 wide variety of groups well known to those of ordinary skill such as bifunctional and

heterobifunctional aliphatic and aromatic-aliphatic groups, amino acids, etc. Thus,  $L_{1-6}$  and  $L_8$  can be the same or different and include groups such as:



15



Preferably,  $L_{1-6}$  and  $L_8$  are selected from among:



Alternatively, suitable amino acid residues can be selected from any of the known naturally-occurring L- amino acids, e.g., alanine, valine, leucine, etc. and/or a combination thereof, to name but a few. L<sub>1-6</sub> and L<sub>8</sub> can also include a peptide which ranges in size, for instance, from about 2 to about 10 amino acid residues.

5 Derivatives and analogs of the naturally occurring amino acids, as well as various art-known non-naturally occurring amino acids (D or L), hydrophobic or non-hydrophobic, are also contemplated to be within the scope of the invention.

### 3. Z Moieties and Their Function

10 In one aspect of the invention, Z and Z' are L<sub>7</sub>-C(=Y<sub>12</sub>) wherein L<sub>7</sub> is a bifunctional linker selected from among the group which defines L<sub>1-6</sub>, and Y<sub>12</sub> is selected from among the same groups as that which defines Y<sub>1</sub>. In this aspect of the invention, the Z group serves as the linkage between the ADA and the remainder of the polymer delivery system. In other aspects of the invention, Z is a moiety that is actively transported into a target cell, a  
15 hydrophobic moiety, and combinations thereof. The Z' when present can serve as a bifunctional linker, a moiety that is actively transported into a target cell, a hydrophobic moiety, and combinations thereof.

In this aspect of the invention, the releasable polymer systems are prepared so that *in vivo* hydrolysis cleaves the polymer from the ADA and releases the enzyme into the  
20 extracellular fluid, while still linked to the Z moiety. For example, some potential Z-B combinations are leucine-ADA and Gly-Phe-Leu-Gly-ADA.

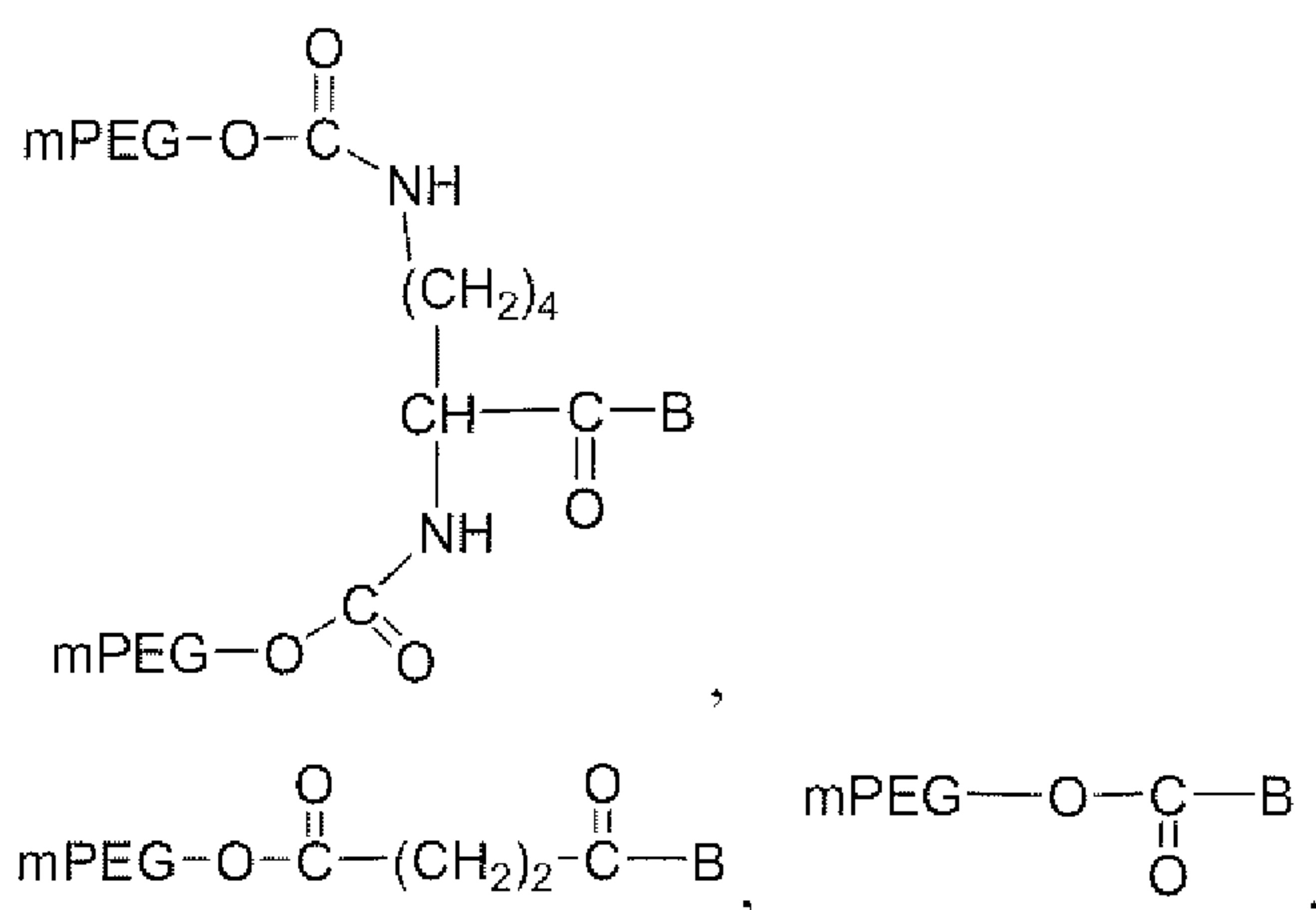
#### B. Preparation of ADA Conjugates

For purposes of illustration, suitable conjugation reactions include reacting ADA with  
25 a suitably activated polymer system described herein. The reaction is preferably carried out using conditions well known to those of ordinary skill for protein modification, including the use of a PBS buffered system, etc. with the pH in the range of about 6.5-8.5. It is contemplated that in most instances, an excess of the activated polymer will be reacted with the ADA.

30 Reactions of this sort will often result in the formation of conjugates containing one or more polymers attached to the ADA. As will be appreciated, it will often be desirable to

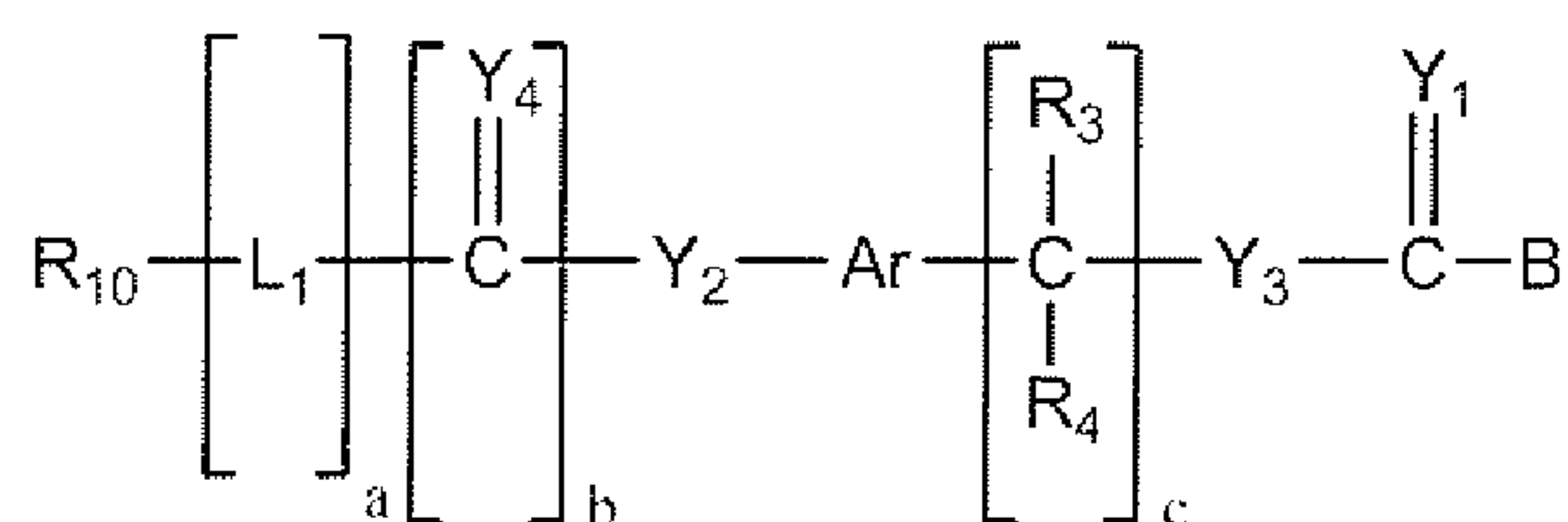


isolate the various fractions and to provide a more homogenous product. In most aspects of the invention, the reaction mixture is collected, loaded onto a suitable column resin and the desired fractions are sequentially eluted off with increasing levels of buffer. Fractions are analyzed by suitable analytical tools to determine the purity of the conjugated protein before being processed further. Regardless of the synthesis route and activated polymer selected, the conjugates will conform to Formula (I) as defined herein. Some of the preferred conjugates which result from the synthetic techniques described herein include:



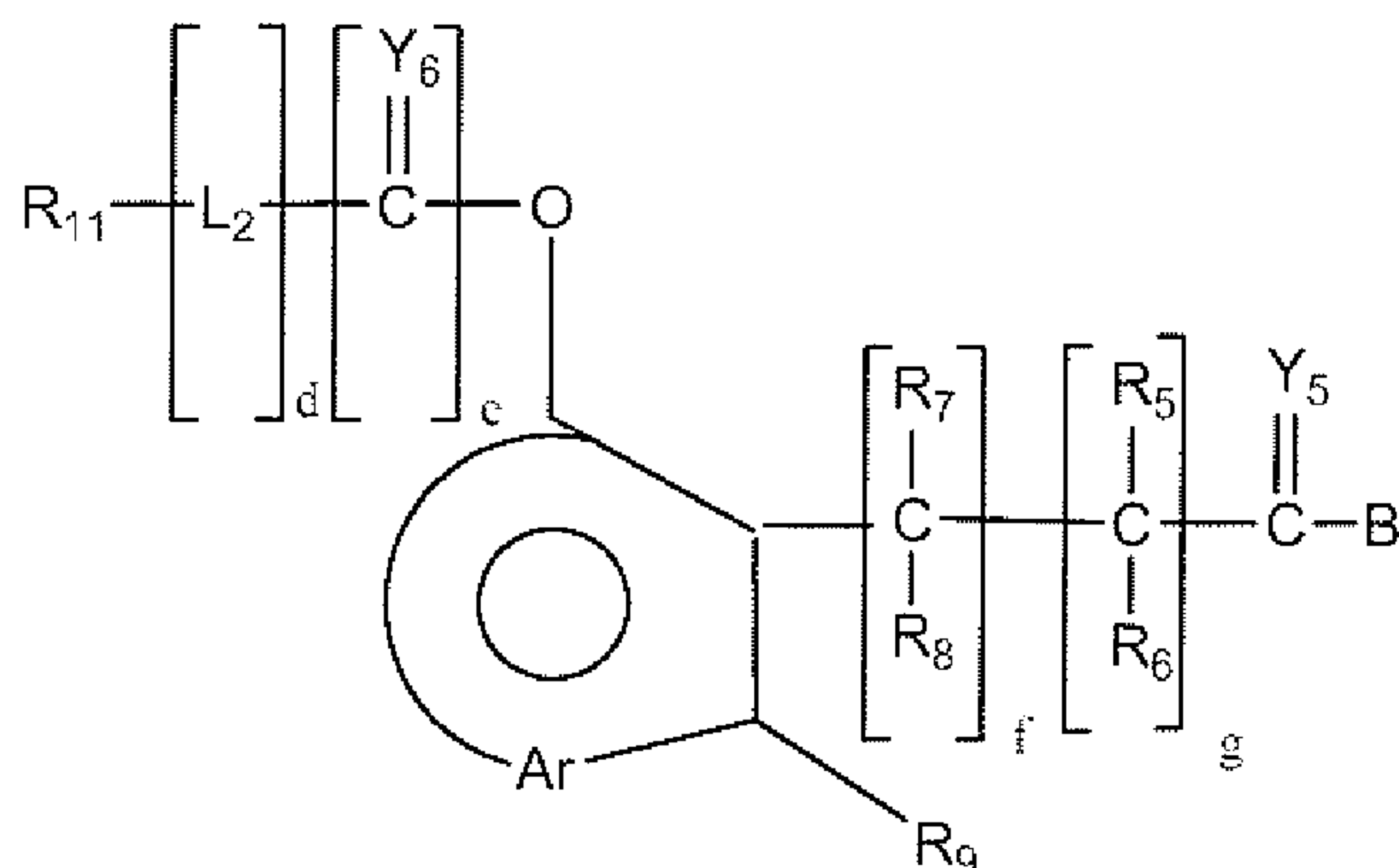
10 wherein B is ADA.

Still further conjugates made in accordance with the present invention include:



wherein all variables are the same as that set forth above and B is ADA.

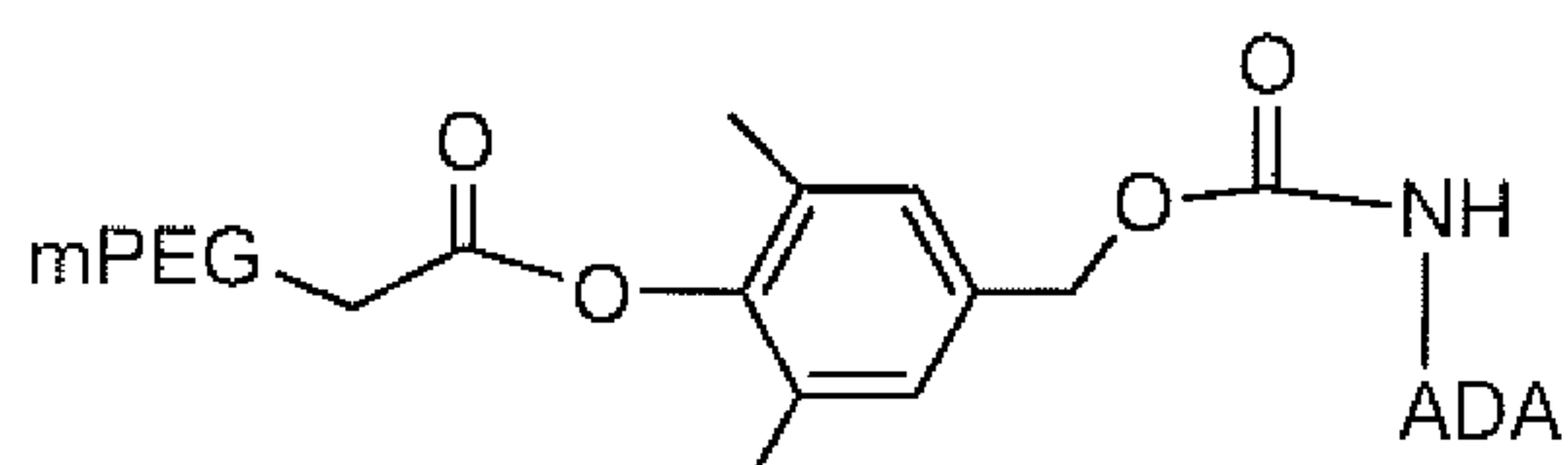
Further conjugates include:



15

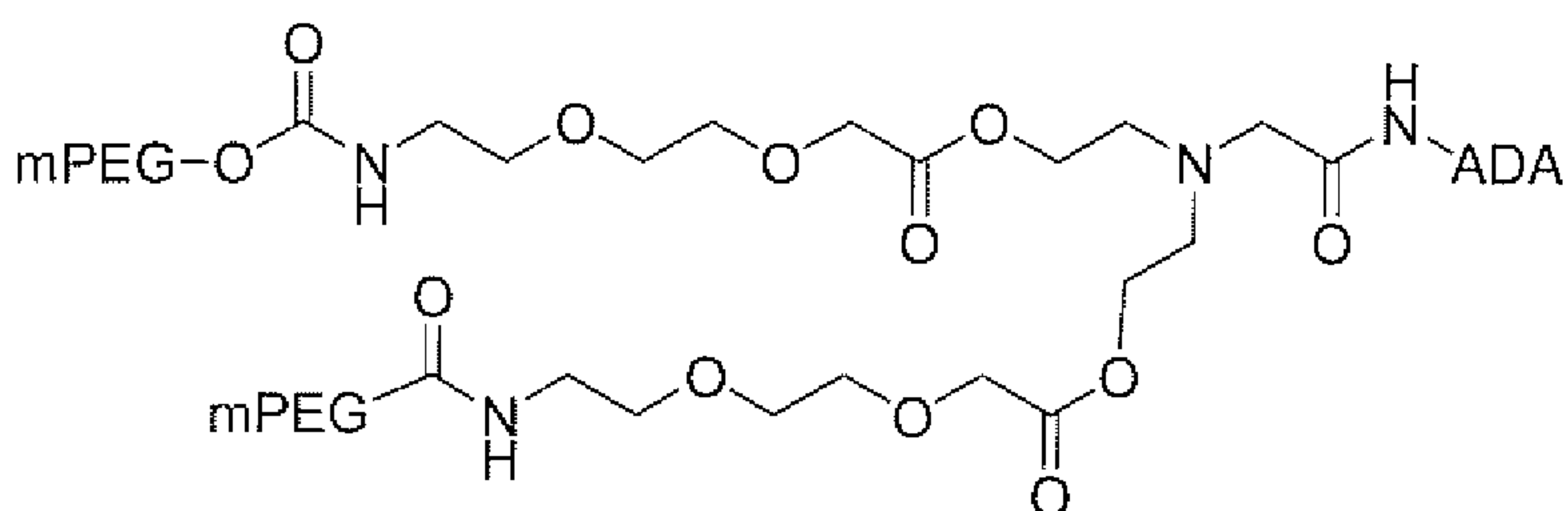
wherein B is ADA.

A particularly preferred conjugate is:

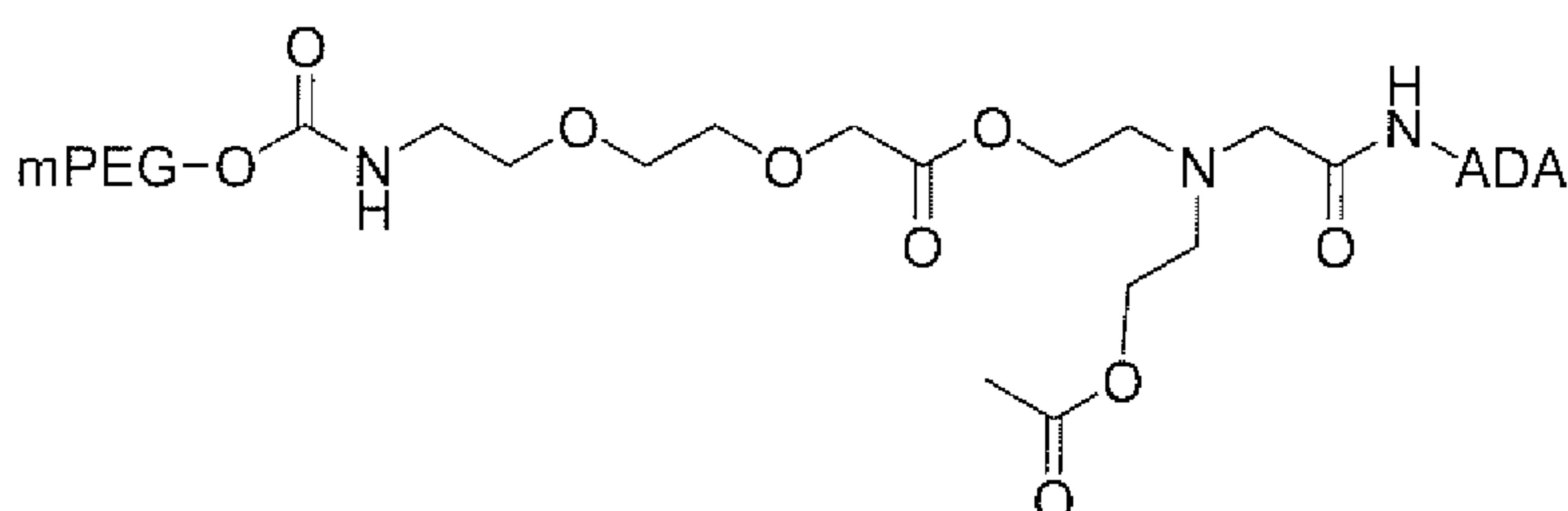


wherein the molecular weight of the mPEG is from about 4,000 to about 45,000.

5 When the bicine-based polymer systems are used, two preferred conjugates are:

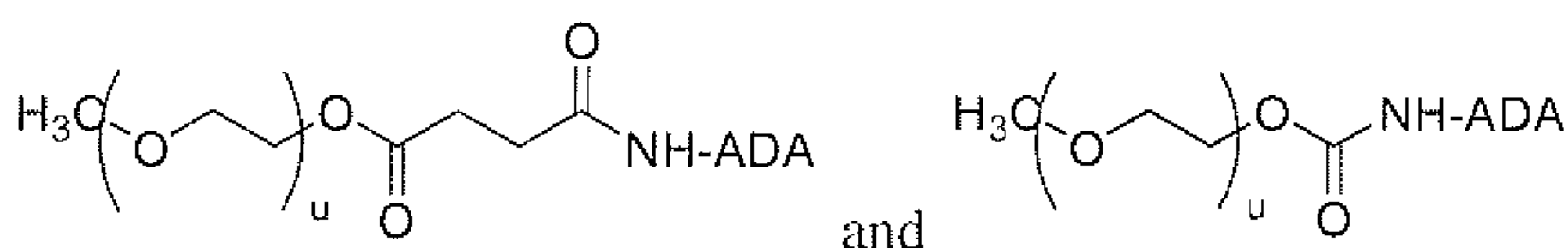


and



wherein the molecular weights of the mPEG are the same as above.

More preferred conjugates include



10

It is noted that PEGylation of ADA will be empirically optimized for total PEG attachments per protein, PEG polymer size, and PEG linker design. Key characteristics of the PEGylated ADA for evaluation of PEGylation optimization include both in vitro assays (*e.g.*, enzyme activity and stability) and in vivo assays (*e.g.*, pharmacokinetics and pharmacodynamics).

15

### C. Tumors To Be Treated

The inventive methods are applicable to treating all types of tumors, including cancers, that are susceptible to a reduction in adenosine or deoxyadenosine levels in the blood

and/or tissues of a human or animal to be treated. Broadly, these include tumors of the blood as well as solid tumors. Among solid tumors are included those that are suppressed when reduced levels of adenosine allow the patient immune system to more effectively suppress the tumor and/or tumors that are suppressed when reduced levels of adenosine inhibit the blood supply, e.g., in already hypoxic tumors.

More preferably, tumors susceptible to treatment by the inventive methods are solid tumors, which benefit from the additional effect of a reduction in angiogenic stimulation, that accompanies a reduction in tissue levels of adenosine.

Tumors to be treated include, simply by way of example, those that originate in the immune system, skeletal system, muscles and heart, breast, gastrointestinal tract, central and peripheral nervous system, renal system, reproductive system, respiratory system, skin, connective tissue systems, including joints, fatty tissues, circulatory system, including blood vessel walls, and the like.

Tumors of the skeletal system include, e.g., both sarcomas and blastomas such as osteosarcoma, chondrosarcoma, chondroblastoma, etc. Muscle and heart tumors include tumors of both skeletal and smooth muscles, e.g., leiomyomas (benign tumors of smooth muscle), leiomyosarcomas, rhabdomyomas (benign tumors of skeletal muscle), rhabdomyosarcomas, cardiac sarcoma, etc. Tumors of the gastrointestinal tract include e.g., tumors of the mouth, esophagus, stomach, small intestine, colon and colorectal tumors, as well as tumors of gastrointestinal secretory organs such as salivary glands, liver, pancreas, the biliary tract and the like.

Tumors of the central nervous system include tumors of the brain, retina, and spinal cord, and can also originate in associated connective tissue, bone, blood vessels or nervous tissue. Tumors of the peripheral nervous system are also contemplated to be treated. In addition, tumors of the peripheral nervous system include malignant peripheral nerve sheath tumors.

Tumors of the renal system include those of the kidneys, e.g., renal cell carcinoma, as well as tumors of the ureters and bladder. Tumors of the reproductive system include tumors of the cervix, uterus, ovary, prostate, testes and related secretory glands. Tumors of the immune system include both blood based and solid tumors, including lymphomas, e.g., both Hodgkin's and non-Hodgkin's.



Tumors of the respiratory system include tumors of the nasal passages, bronchi and lungs. Tumors of the breast include, e.g., both lobular and ductal carcinoma.

Particularly common types of malignant tumors to be treated include, simply by way of example: prostate cancer, lung cancer, breast cancer, colorectal cancer, bladder cancer, 5 pancreatic cancer, endometrial cancer, ovarian cancer, cutaneous melanoma, lymphomas, non-Hodgkin lymphoma, pancreatic cancer, neuroblastoma, Wilms' tumor, rhabdomyosarcoma (arising from muscle), retinoblastoma, osteosarcoma and Ewing's sarcoma, to name but a few.

#### 10 **D. Dose Selection**

The artisan will appreciate that, in a clinical setting, the dosage of Adagen<sup>®</sup> is individualized depending upon the clinical response of the tumor and the side effect profile of an individual patient, whether animal or human. In the example study provided herein below, the highest dose is the maximum feasible dose that is tolerated. Adagen<sup>®</sup> is commercially 15 supplied as 250U/mL. This translates to 2000U/kg for an approximate 25g mouse injected with 0.2ml of Adagen<sup>®</sup>. The lowest dose employed in the examples studies provided hereinbelow approximates the clinical human dose. The recommended dosing schedule in treating human SCID patients is 10 U/kg for the first dose, 15 U/kg for the second dose, and 20 U/kg for the third dose. Further increases of 5 U/kg/week are permitted, up to a maximum 20 single dose of 30 U/kg. The dose in the below exemplified protocol (100 U/kg) is the mouse equivalent dose of approximately 12 U/kg clinical child dose.

The dose based on the amount of enzyme will range from, for example, about 0.10U/kg through about 30 U/kg, or higher, preferably from about 0.5 U/kg through about 20 U/kg, and more preferably from about 0.5 U/kg through about 12U/kg (i.e. per kg of patient 25 body weight) such as from about 0.5U/kg through about 5U/kg. A total weekly dose can be up to 40U/kg, or more, as tolerated by the recipient. Further increases of 5 U/kg/week are permitted, up to a maximum single dose of 30 U/kg, or more, as tolerated by the recipient. In general, following weekly injections of ADAGEN<sup>®</sup> at 15U/kg, the average trough level of ADA activity in plasma is between 20 and 25  $\mu\text{mol/hr/mL}$ .

30 Of course, the artisan will appreciate that the dose of polymer-conjugated ADA can also be adjusted for the particular polymer size, linker chemistry, and valency. For example,

the dosing regimen for a polymer conjugate comprising two or four ADA enzymes per polymer will be adjusted according to the units of ADA per ml of solution of any particular polymer conjugate of ADA.

In providing the ADA or ADA PEG-conjugate by injection, the optimal dose range can be adjusted by monitoring adenosine levels in plasma. It is generally desirable to provide the recipient with a dosage that will maintain plasma ADA activity (trough levels) in the range of from about 10 to 100  $\mu\text{mol/hr/mL}$ , preferably from about 15 to about 35  $\mu\text{mol/hr/mL}$  (assayed at 37°C); and demonstrate a decline in erythrocyte adenosine, i.e., dATP to  $\leq$  about 0.001- 0.057  $\mu\text{mol/mL}$ , preferably about 0.005- about 0.015  $\mu\text{mol/mL}$  in packed erythrocytes, or  $\leq$  about 1% of the total erythrocyte adenosine (i.e., ATP + dATP content), with a normal adenosine level, as measured in a pre-injection sample. The normal value of dATP is below about 0.001  $\mu\text{mol/mL}$ .

Details of ADA dosage information are described in the prescription insert for ADAGEN<sup>®</sup> (Enzon, Inc.).

## EXAMPLES

The following examples serve to provide further appreciation of the invention but are not meant in any way to restrict the effective scope of the invention.

### EXAMPLE 1

#### ANTITUMOR EFFICACY OF ADAGEN<sup>®</sup> IN DU145 HUMAN PROSTATE TUMOR XENOGRAFT MODEL

##### A) TEST SYSTEM

25	<u>Species:</u>	Mouse, Mus musculus
	<u>Strain:</u>	Athymic nude
	<u>Supplier:</u>	Harlan-Sprague Dawley
30	<u>Sex:</u>	Female
	<u>Mean Initial Weight:</u>	27.2 g
35	<u>No. on Study:</u>	40

Acclimation Period: 7 days following arrival

Identification: Cage number and ear punch

5

## B) METHODS

### Experimental Design:

DU 145 human prostate cancer cells were obtained from the American Type Culture Collection (ATTC), Manassas, VA. Tumors were established in nude mice by subcutaneous injection of  $2.0 \times 10^6$  DU 145 cells/mouse into the right axillary flank. Tumor growth was monitored twice weekly and measured once palpable. When tumors reached an average volume of  $78 \text{ mm}^3$ , mice were divided into experimental groups (8/group). Mice were treated with Adagen<sup>®</sup> at either 2000, 500, or 100 IU/kg twice weekly for 5 weeks. As a positive control, mice received Avastin<sup>®</sup> (Bevacizumab, an anti-VEGF monoclonal antibody) at 5mg/kg dose at the same frequency as Adagen<sup>®</sup>. The experimental groups were set up as shown in Table 1, below. The first day of dosing was designated as Day 1. The tumor volume for each mouse was determined by measuring two dimensions with calipers and calculated using the formula: tumor volume = (length x width<sup>2</sup>)/2. Mouse weight and tumor size were measured at the beginning of the study and twice weekly throughout the 8-week period.

20

**TABLE 1**

Group #	Group	# animals (n)	Dose (IU/kg)	Route of injection	Dosing schedule
1	Control	8	Saline	i.p.	Twice Weekly x 5
2	Adagen <sup>®</sup>	8	2000	i.p.	Twice Weekly x 5
3	Adagen <sup>®</sup>	8	500	i.p.	Twice Weekly x 5
4	Adagen <sup>®</sup>	8	100	i.p.	Twice Weekly x 5
5	Avastin <sup>®</sup>	8	5mg/kg	i.p.	Twice Weekly x 5

### Dose Selection:

Adagen<sup>®</sup> or Avastin<sup>®</sup> was administered by the intraperitoneal ("i.p.") route twice weekly for five weeks (total: 10 doses).

25

Adagen<sup>®</sup>: Lot Number: NV0604, Concentration: 229 IU/ml

Avastin<sup>®</sup>: Lot Number: M66781, Concentration: 25 mg/ml

Dose Calculations were based on body weight taken on day 1.



Clinical Examinations:

The mice were examined visually upon arrival. Thereafter, mice were individually examined twice a week following initial tumor palpation, for clinical signs, general behavioral changes, and monitored for body weight. Any death and clinical sign were recorded. Food and water consumption was not monitored. Mice bearing tumors that showed open necrotic lesions were sacrificed. Mice losing more than 20% of body weight were also humanely sacrificed.

Statistical Analysis:

Differences in % change in tumor volume between various treatments were compared using one way analysis of variance. All pairwise multiple comparisons were made using the Holm-Sidak method.

**C) RESULTS**15 Definitions of the terms used:

(a) % of Initial tumor volume:  $(\text{Tumor volume on any given day} / \text{Tumor volume on Day 1}) \times 100$

(b) % change in tumor volume:  $[(\text{Tumor volume on any given day} - \text{Tumor volume on Day 1}) / \text{Tumor volume on day 1}] \times 100$

20 (c) % Tumor growth inhibition (TGI):  $[(\text{Mean tumor volume of control group} - \text{Mean tumor volume of treatment group}) / \text{Mean tumor volume of control group}] \times 100$

(d) Regression of tumor is defined as negative tumor volume compared to day 1

(e) Cure is defined as complete absence of tumor as observed from naked human eye.

25 The average tumor size at the beginning of the study was  $78 \text{ mm}^3$ . The average body weight at the beginning of the study was 27.20g. Mice in all the groups gained weight and by the end of the study the weights of mice in the various groups were increased by 20 to 25% of their pretreatment weight. The study was terminated on day 57, when a majority of the animals either had ulcerated tumors or had a tumor volume of over  $1500 \text{ mm}^3$ .

30 Tables 2 and 3, below, summarize the final results seen on day 49 of the study (last day of 100% survival of control animals), and provide a group survival comparison between

the Adagen<sup>®</sup> treated animals and the Avastin<sup>®</sup> treated animals. Tumors in the control group grew steadily throughout the study. On day 49, the mean tumor volume size was 783.1 ( $\pm$  556.2) mm<sup>3</sup>. The percent change in tumor growth was 833.3 % ( $\pm$  622.7). Treatment with Adagen<sup>®</sup> at all the three dose levels was effective in inhibiting tumor growth. It should be noted that for all the treatment groups, the tumors grew at a slow rate until the last day of dosing (day 33), after which the tumors had a faster rate of growth (data not shown).

Consistent with this observation is the fact that the % change in tumor volume at all of the three dose levels of Adagen<sup>®</sup> was significantly different from that of the control group until day 36 (P<0.05) (data not shown).

This suggests that Adagen<sup>®</sup> has a cytostatic effect on tumor growth. Although the % change in tumor volume was not statistically different from that of controls after day 36 until the end of the study, treatment with Adagen<sup>®</sup> resulted in detectable tumor growth inhibition. In particular, tumors treated with 2000IU/kg Adagen<sup>®</sup> had average tumor volume of 343.0 ( $\pm$ 249.8) mm<sup>3</sup> on day 49. The average change in tumor volume was 424.2 % ( $\pm$ 360.6).

Adagen<sup>®</sup> at 2000IU/kg showed 56% tumor growth inhibition.

The tumors treated with 500IU/kg Adagen<sup>®</sup> had a mean tumor volume of 696.3 ( $\pm$ 290.4) mm<sup>3</sup> on day 49. The average change in tumor volume was 797.6% ( $\pm$ 492.7), and the change from the initial tumor was 897.6% ( $\pm$ 492.7). Tumor growth inhibition was 11.1%. Tumors in this group had similar tumor growth rates as the other Adagen<sup>®</sup>-treated groups until day 40 after which they grew at a much faster rate. The reason for this discrepancy is unknown.

The tumors treated with 100IU/kg Adagen<sup>®</sup> had a mean tumor volume of 414.8 ( $\pm$ 219.0) mm<sup>3</sup> on day 49. The average change in tumor volume was 489.9% ( $\pm$ 307.0), and the average percent change from the initial tumor was 589.9% ( $\pm$ 307.0). Tumor growth inhibition or TGI was 47.0%.

The therapeutic effect of Adagen<sup>®</sup> reached a maximal effect at the lowest dose of 100IU/kg. It should be noted that 100 IU/kg dose approximates the clinical human dose of Adagen<sup>®</sup> (dose used to treat SCID children). Avastin<sup>®</sup> showed the most effective decrease in tumor size with a tumor growth inhibition or TGI of 92.5%.

In conclusion, treatment with Adagen<sup>®</sup> at all the three dose levels was effective in inhibiting growth of DU145 tumors *in vivo*.

TABLE 2: Final Summary (Day 49)

Group No.	Compound	Final Tumor Volume Means	Final Tumor Volume Medians	% Change in Tumor Volume	% Initial Tumor Volume	% Initial Tumor Volume	# of Regressions	# Cured	% Survival
1	Saline	783.1 (556.2)	680.0	833.3 (622.7)	933.3 (622.7)	-	0	0	62.5
2	Adagen (2000 IU/kg)	343.0 (249.8)	283.8	424.2 (360.6)	524.2 (360.6)	56.2	0	0	75.0
3	Adagen (500 IU/kg)	696.3 (290.4)	766.2	797.6 (492.7)	897.6 (492.7)	11.1	0	0	75.0
4	Adagen (100 IU/kg)	414.8 (219.0)	349.8	489.9 (307.0)	589.9 (307.0)	47.0	0	0	75.0
5	Avastin (5 mg/kg)	58.5 (36.1)	49.3	-15.7 (59.6)	84.3 (59.6)	92.5	7/8	0	87.5

# regression, # cured and % survival data are from day 56.

Tumor volume means, medians, % change in tumor volume, % Initial tumor volume and % Tumor growth inhibition data are from day 49.



**TABLE 3: Group Survival Comparison (# Animal Live)**

Group	1	5	8	12	15	19	22	26	29	33	36	40	43	49	51	55	57
1:Saline	8	8	8	8	8	8	8	8	8	8	8	8	8	5 <sup>a</sup>	5	5	5
2:Adagen (2000U/kg)	8	8	8	8	8	8	8	8	8	8	8	7 <sup>c</sup>	7	6 <sup>h</sup>	6	6	6
3:Adagen (500U/kg)	8	8	8	8	8	8	8	8	8	8	8	7 <sup>c</sup>	7	6 <sup>h</sup>	6	5 <sup>i</sup>	5
4:Adagen (100U/kg)	8	8	8	8	8	8	8	8	8	8	8	6 <sup>b</sup>	6	6	6	5 <sup>i</sup>	5
5:Avastin (5mg/kg)	8	8	8	8	8	8	8	8	8	8	8	8	8	7 <sup>h</sup>	7	7	7

<sup>a</sup> Two animals were sacrificed due to tumor sizes in excess of 1500mm<sup>3</sup>. One animal sacrificed due to an ulcerated tumor.

<sup>c</sup> Animal sacrificed due to an ulcerated tumor on day 40.

<sup>h</sup> Animal sacrificed due to an ulcerated tumor on day 49.

<sup>i</sup> Animal sacrificed due to an ulcerated tumor on day 55.

<sup>b</sup> Two animals were sacrificed due to ulcerated tumors on day 49.

**EXAMPLE 2****ANTITUMOR EFFICACY OF ADAGEN® IN SK-OV-3  
HUMAN OVARIAN TUMOR XENOGRAFT MODEL****A) TEST SYSTEM**

<u>Species:</u>	Mice, Mus musculus
<u>Strain:</u>	Athymic nude
<u>Supplier:</u>	Harlan-Sprague Dawley
<u>Sex:</u>	Female
<u>Mean Initial Weight:</u>	22.18 g
<u>No. on Study:</u>	54
<u>Acclimation Period:</u>	7 days following arrival

**B) METHODS**Experimental Design:

SK-OV-3 human ovarian adenocarcinoma tumors were established in nude mice by subcutaneous injection of  $3 \times 10^6$  cells/mouse into the right axillary flank. Tumor growth was monitored twice weekly and measured once palpable. The tumor volume for each mouse was determined by measuring two dimensions with calipers and calculated using the formula: tumor volume = (length x width<sup>2</sup>)/2). When tumors reach an average volume of 90 mm<sup>3</sup>, the mice were

divided into experimental groups (9/group). The experimental groups were set up as shown in the table below. The first day of dosing was designated as Day 1. Mouse weight and tumor size were measured at the beginning of the study and twice weekly until termination of the study. The study was terminated after approximately 7 weeks (52 days), when majority of animals had large or ulcerated tumor masses.

TABLE 4

Group #	Group	# mice (n)	Dose (U/kg)	Route of injection	Dosing schedule
1	Control	9	saline	i.p.	Twice weekly x 5
2	Adagen <sup>®</sup>	9	2000	i.p.	Twice weekly x 5
3	Adagen <sup>®</sup>	9	500	i.p.	Twice weekly x 5
4	Adagen <sup>®</sup>	9	100	i.p.	Twice weekly x 5
5	Avastin <sup>®</sup>	9	100 ug/mouse	i.p.	Twice weekly x 5
6	Native ADA	9	2000	i.p.	Twice weekly x 5

#### Dosing Regimen:

Adagen<sup>®</sup>, Avastin<sup>®</sup>, or Native ADA was administered intravenously twice weekly for five weeks (total: 10 doses).

#### Test Articles:

Adagen<sup>®</sup>: Lot Number: NV0604, Concentration: 229 IU/ml

Avastin<sup>®</sup>: Lot Number: M66781, Concentration: 25 mg/ml

Native ADA: Lot Number: 06-0315-111

#### Dose Calculations:

Based on body weight taken on day 1.

#### Clinical Examinations:

The mice were examined visually upon arrival. Thereafter, mice were individually examined twice a week following initial tumor palpation, for clinical signs, general behavioral changes, and monitored for body weight. Any death and clinical sign were recorded. Food and water consumption was not monitored. Mice bearing tumors that showed open necrotic lesions were sacrificed. Mice losing more than 20% of body weight were also humanely sacrificed.

Statistical Analysis:

Differences in % change in tumor volume between various treatments were compared using one-way analysis of variance. All pairwise multiple comparisons were made using the Tukey-Kramer method.

**C) RESULTS**Definitions of the terms used:

- (a) % of Initial tumor volume:  $(\text{Tumor volume on any given day} / \text{Tumor volume on Day 1}) \times 100$
- (b) % change in tumor volume:  $[(\text{Tumor volume on any given day} - \text{Tumor volume on Day 1}) / \text{Tumor volume on day 1}] \times 100$
- (c) % Tumor growth inhibition (TGI):  $[(\text{Mean tumor volume of control group} - \text{Mean tumor volume of treatment group}) / \text{Mean tumor volume of control group}] \times 100$
- (d) Regression of tumor is defined as negative tumor volume compared to day 1
- (e) Cure is defined as complete absence of tumor as observed from naked human eye

The average tumor size at the beginning of the study was  $90 \text{ mm}^3$ . The average body weight at the beginning of the study was 22.2g. The mean body weight in all the groups did not change significantly throughout the study. The study was terminated on day 52. Four of the mice were sacrificed due to tumors growing over  $1500 \text{ mm}^3$ .

Table 5, below, gives the final summary of results as on day 32 (last day of 66% survival of control animals). Tumors in the control group grew steadily throughout the study. On day 32, the mean tumor volume size was  $754.9 (\pm 700.7) \text{ mm}^3$ . The percent change in tumor growth was  $693.2 \% (\pm 673.6) \text{ mm}^3$ .



TABLE 5: Final Summary (Day 32)

Group No.	Compound	Final Tumor Volume Means	Final Tumor Volume Medians	% Change in Tumor Volume	% Initial Tumor Volume	% Initial Tumor Volume	# of Regressions	# Cured	% Survival
1	Saline	754.9 (700.7)	485.5	693.2 (673.6)	793.2 (673.6)	-	0	0	66
2	Adagen <sup>®</sup> (2000 IU/kg)	437.7 (122.2)	446.4	402.1 (177.7)	502.1 (177.7)	42.0	0	0	100
3	Adagen <sup>®</sup> (500 IU/kg)	471.1 (60.0)	465.8	421.3 (102.2)	521.3 (102.2)	37.6	0	0	89
4	Adagen <sup>®</sup> (100 IU/kg)	497.7 (152.6)	478.9	450.7 (172.7)	550.7 (172.7)	34.1	0	0	100
5	Avastin <sup>®</sup> (5 mg/kg)	210.2 (65.9)	198.6	161.6 (88.3)	261.6 (88.3)	72.2	0	0	100
6	Native ADA (2000 IU/kg)	356.6 (213.0)	329.7	340.1 (239.1)	440.1 (239.1)	52.8	0	0	77

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Tumors treated with 2000IU/kg Adagen<sup>®</sup> had average tumor volume of 437.7 ( $\pm$ 122.2) mm<sup>3</sup> on day 32. The average change in tumor volume was 402.1 % ( $\pm$ 177.7). Adagen<sup>®</sup> at 2000IU/kg showed 42% tumor growth inhibition.

The tumors treated with 500IU/kg Adagen<sup>®</sup> had a mean tumor volume of 471.1 ( $\pm$ 60.0) mm<sup>3</sup> on day 32. The average change in tumor volume was 421.3% ( $\pm$ 102.2), and the change from the initial tumor was 521.3% ( $\pm$ 102.2). Tumor growth inhibition was 37.6%.

The tumors treated with 100IU/kg Adagen<sup>®</sup> had a mean tumor volume of 497.7 ( $\pm$ 152.6) mm<sup>3</sup> on day 32. The average change in tumor volume was 450.7% ( $\pm$ 172.7), and the average percent change from the initial tumor was 550.7% ( $\pm$ 172.7). Tumor growth inhibition was 34.1%.

The tumors treated with 2000IU/kg Native ADA had a mean tumor volume of 356.6 (213.0)mm<sup>3</sup> on study day 32. The average change in tumor volume was 340.1% ( $\pm$ 210.0), and the average percent change from the initial tumor was 440.1% (239.1). Tumor growth inhibition was 52.8%.

Treatment with Adagen at either dose level was commensurate with the results from controls. However, Adagen at each dose level produced TGI as noted above.

The therapeutic effect of Adagen<sup>®</sup> reached a maximal effect at the lowest dose of 100IU/kg. It should be noted that 100IU/kg dose approximates with the clinical human dose of Adagen<sup>®</sup> (dose used to treat SCID children). Avastin<sup>®</sup>, serving as a positive control in this study, showed the most effective decrease in tumor size with a TGI of 72%.

In conclusion, treatment with Adagen<sup>®</sup> at 100, 500 or 2000 IU/kg resulted in tumor growth inhibition ranging from 34-42%.

Alternative recombinant ADA enzymes for the inventive methods are described, as follows.

### EXAMPLE 3

#### CONSTRUCTION OF *E. COLI* EXPRESSION STRAIN EXPRESSING RECOMBINANT HUMAN ADA WITH A CYS TO SER CHANGE AT POSITION 74 OF THE MATURE PROTEIN

The reported 363 amino acid sequence of human adenosine deaminase (GenBank NP\_000013) was analyzed for the presence of cysteine codons.



Five positions in the mature (N-terminal Met is cleaved) polypeptide encode cysteine (C74, C152, C153, C168, C261). In the designed and modified gene expressing human ADA, only one of these five cysteine codons (Cysteine 74, TGC) was changed to a serine codon (TCC) (this is position 75 in the translated protein). The defined polypeptide sequence (see SEQ ID NO: 3) was provided to Blue Heron Corporation (Bothell, Washington, U.S.A.) for whole gene synthesis of a new gene having codons optimized for expression in *E. coli*, using standard chemical synthesis of overlapping oligonucleotide segments. In brief, the sequence was optimized for bacterial expression by following the standard bacterial codon usage for *Escherichia coli* K12, using the codon data described by Grantham R. *et al.*; 1981; "Codon catalogue usage in genome strategy modulated for gene expressivity," *Nucleic Acid Res.* 9:r43-r47, and Lathe, R. 1985; "Synthetic oligonucleotide probes deduced from amino acid sequence data, Theoretical and practical considerations." *J. Mol Biol*; 183:1-12.

The corresponding RNA sequence was then analyzed for the formation of hairpin structure or loop formation and was subjected to minimum free energy calculations. The flanking restriction sites, *NdeI* and *BamHI* were included at the termini of the gene. Following digestion of the synthetic DNA with the restriction enzymes *NdeI* and *BamHI*, the 1.1 kilobase gene was ligated via T4 DNA ligase into the plasmid vector pET-28a (Novagen Corporation), which had also been digested with these two enzymes. The recombinant plasmid was introduced into *E. coli* strain BLR (DE3) or HMS174 (DE3) by electroporation using a BTX Electro Cell Manipulator 600 according to the manufacturer's instructions. The transformation mixture was plated on LB agar plates containing kanamycin (15 µg/ml) in order to allow for selection of colonies containing the plasmid pET-28a/ADAcysSer (designated ADAc75s/pET28a: BLR(DE3) or ADAc75s/pET28a:HMS174(DE3)). The ADA variant gene nucleotide sequence was verified by DNA sequence analysis with a ABI Prism 310 Genetic Analyzer using Big Dye Terminators. The DNA sequence encoding the Ser<sub>74</sub>-rhADA open reading frame is according to SEQ ID NO: 4.

Isolated colonies were further purified by plating and analyzed for IPTG inducible gene expression in LB medium by standard methods such as those described in Novagen pET System Manual Ninth Edition.

Several induction parameters were examined including time, temperature and inducer concentration. A preferred condition was induction with 50 µM IPTG for 12 hrs at 25 °C, which



allowed high level production of ADA within the cytoplasm of the host bacteria at about 20% of total cell protein. The expressed ADA protein was confirmed on SDS PAGE analysis to exhibit the correct molecular weight of approximately 40 kDa (data not shown).

#### EXAMPLE 4

### **CONSTRUCTION OF *E. COLI* EXPRESSION STRAIN EXPRESSING RECOMBINANT BOVINE ADA WITH A CYS TO SER CHANGE AT POSITION 74 OF THE MATURE PROTEIN**

The purified mature ADA protein derived from bovine intestinal preparations is a 356 amino acid protein lacking the N-terminal methionine and also lacking the final six C-terminal residues predicted from the cDNA sequence (GenBank NP\_776312).

The bovine ADA amino acid sequence was analyzed for the presence of cysteine codons. Five positions in the mature polypeptide encode cysteine (C74, C152, C153, C168, C261). In the designed and modified bovine ADA synthetic gene, only one of these five cysteine positions (cysteine 74) was changed to a serine residue. This was performed by inserting a serine codon (TCC) in place of the normal cysteine codon at position 74 of the mature polypeptide (or position 75 of the translation product). The gene was also codon optimized for expression in *E. coli*.

In brief, the defined polypeptide sequence (see SEQ ID NO: 1) was provided to BioCatalytics Inc. for whole gene synthesis of a new gene having codons optimized for expression in *E. coli*, using their methods that include chemical synthesis of overlapping oligonucleotide segments. The BioCatalytics methods are described in greater detail by U.S. Patent No. 6,366,860.

Bovine ADA expression was investigated in several expression systems. For example, the flanking restriction sites, *NdeI* and *BamHI* were included at the termini of the gene. Following digestion of the synthetic DNA with the restriction enzymes *NdeI* and *BamHI*, the 1.1 kilobase gene was ligated via T4 DNA ligase into the plasmid vector pET-9d (Novagen Corporation), which had also been digested with these two enzymes. The recombinant plasmid was introduced into *E. coli* strain BLR (DE3) or HMS174 (DE3) by electroporation using a BTX Electro Cell Manipulator 600 according to the manufacturer's instructions. The transformation mixture was plated on LB agar plates containing kanamycin (15 µg/ml) to allow for selection of

colonies containing the plasmid pET-9d/bADA (designated bADA/pET9d: BLR(DE3) or bADA/pET9d:HMS174(DE3)). The ADA variant gene nucleotide sequence was verified by DNA sequence analysis with a ABI Prism 310 Genetic Analyzer using Big Dye Terminators. The open reading frame of the DNA is shown by SEQ ID NO: 2.

Isolated colonies were further purified by plating and analyzed for IPTG inducible gene expression in LB medium by standard methods such as those described in Novagen pET System Manual Ninth Edition. Several induction parameters were examined including time, temperature and inducer concentration. A preferred condition was induction with 0.3 % lactose for 12 hrs at 37 °C, which allowed high level production of ADA within the cytoplasm of the host bacteria at about 20% of total cell protein. The ADA product was confirmed on SDS PAGE analysis to exhibit the correct molecular weight of approximately 40 kDa.

## EXAMPLE 5

### PURIFICATION OF RECOMBINANT HUMAN MUTEIN ADA PROTEIN

The purification of rhADA was carried out in a 3 chromatographic protocol developed by Enzon. Bacterial fermentation was conducted for *E. coli* expressing the rhADA protein from a synthetic gene on plasmid pET28a (Novagen) in host cell HMS174(DE3). Rifampicin (200 µg/ml) and kanamycin (30 µg/ml) were included in a minimal glycerol medium supplemented with yeast extract (30 g/l) and the cells were grown at 28 °C to an OD<sub>600</sub> of 11 when the inducer IPTG was added to 5 mM final concentration. After 40 hours (OD<sub>600</sub> ~ 110), the cells were harvested by centrifugation and frozen at -20 °C. Briefly, thawed cell paste (50 g) was re-suspended in 1800 ml buffer of 10 mM Tris, 1mM DTT, pH 8.0, and homogenized at 1200 RPM for 10 seconds with Tempest Virtis (Sentry™, Microprocessor, Boston, MA). This suspension was passed through a stainless steel mesh (Opening micrometer 250µ, No.60, W.S Tyler) to remove big particles. The homogenous cell suspension was microfluidized for 3 cycles at 15,000 psi (unit was ice-bathed) (Micro Fluidizer, Microfluidics Corp., Model# 110Y, Boston, MA). At the end of micro fluidization, 200 ml of the same buffer as above was used to rinse the unit and this solution was combined with the above suspension. The soluble protein from cell lysates was extracted by centrifugation at 16,000 rpm for 40 minutes at 4° C (Sorvall RC 5C plus, rotor SLA-1000). The supernatant was collected carefully to avoid unwanted mixing. The



pH was adjusted to 8.0, and 1 mM MgCl<sub>2</sub> and 20 mg/mL DNase were added and incubated at room temperature for 2 hours. The pH was then adjusted to 6.5 with 1 N HCl. A second centrifugation was conducted as above, the supernatant collected, and adjusted to 2 mM EDTA, followed by filtration on Nalgene (90 mm filter unit). The volume of the filtered supernatant was 500 ml, total protein concentration by BCA method was 8.5 mg/ml.

The cell extract (100 ml) was adjusted to pH 7.2, 4.5 mS/cm and loaded onto HiTrap DEAE ff at 20 mM Bis-Tris, 20 mM NaCl, pH 6.5 and eluted with 20 mM Bis-Tris, 500 mM NaCl, pH 6.5. The peak fractions were identified by enzyme assay and SDS-PAGE and adjusted to 1.5 M ammonium sulfate in 20 mM NaHPO<sub>4</sub>, pH 6.5 and loaded onto a HiTrap Phenyl ff column. The protein was eluted with a gradient of load buffer and 20 mM NaHPO<sub>4</sub>, pH 6.5. The peak fraction (55 ml; 0.4 mg/ml) was diafiltered against 20 mM NaHPO<sub>4</sub>, 1 mM EDTA, 1 mM DTT, pH 6.5 and loaded onto HiTrap SP-Sepharose ff and eluted with 20 mM NaHPO<sub>4</sub>, 500 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 6.5. The collected fraction contained purified ADA protein (77 ml; 0.1 mg/ml).

#### **EXAMPLE 6**

#### **PURIFICATION OF RECOMBINANT BOVINE ADA PROTEIN**

The purification of rbADA expressed by the clone of Example 4 was carried out in a 3 chromatographic protocol developed by Enzon. Briefly, thawed cell paste (obtained from Blue Hereon or Biocatalytics, respectively) of 200 g which was stored at -80°C was re-suspended in 1800 ml buffer of 20 mM Bis-Tris, 1mM EDTA, pH 7.4, and homogenized at 1200 RPM for 5 min with Tempest Virtis (Sentry™, Microprocessor, Boston, MA). This suspension was passed through a stainless steel mesh (Opening micrometer 250μ, No.60, W.S Tyler) to removed big particles. The homogenous cell suspension was microfluidized for 3 cycles at 15,000 psi (unit was ice-bathed) (Micro Fluidizer, Microfluidics Corp., Model# 110Y, Boston, MA). At the end of micro fluidization, 200 ml of the same buffer as above was used to rinse the unit and this solution was combined with the above suspension. The soluble protein from cell lysates was extracted by centrifugation at 7100 rpm (12000 x g) for 60 minutes at 4° C (Avanti J-201, Beckman Coulter; Rotor# JLA8.1000). The supernatant was collected carefully to avoid unwanted mixing.



To remove nucleotides in this cell extract, polyethyleneimine (PEI) was added to the above supernatant (final 0.15%, wt/v) and mixed thoroughly by stirring for 10 min. Then left this cell extract at 4° C over night. The precipitant from this over night sample was removed by a centrifugation at 7100 rpm (12000 x g), for 60 minutes at 4° C (Avanti J-201, Beckman Coulter; Rotor# JLA8.1000). Similarly, the supernatant was collected carefully to avoid any unwanted mixing. To help ADA bind to the first column, 10% PEG4600 was added to this cell extract slowly and the pH of this cell extract was adjusted to 6.5 slowly with 1 N NaOH and 1N HCl. This supernatant was centrifuged again at 7100 rpm (12000 x g), for 60 minutes at 4° C (Avanti J-201, Beckman Coulter; Rotor# JLA8.1000) before loaded to the next column.

The cell extract was loaded to a pre-equilibrated Capto Q column (Cat# 17-5316-01, GE Healthcare, Piscataway, NJ. Bed volume 350 ml pre packed in a XK-50 column) with a buffer of 20 mM Bis-Tris, 1 mM EDTA, pH 6.5. Before ADA was eluted off from the column at 80 mM NaCl in the equilibration buffer, elutions at 60 mM and 70 mM NaCl were first performed to remove impurities. The elution profile was analysed by ADA activity, SDS-PAGE analysis, Western Blots, and RP-HPLC.

After Capto Q column, 2 hydrophobic interaction chromatographic purifications were used one by one to further polish the purity of the protein. The first HIC was Octyl Sepharose 4FF (Cat# 17-0946-02, GE Healthcare, Piscataway, NJ). The pool of ADA fractions from Capto Q column was adjusted to 1.5 M  $(\text{NH}_4)_2\text{SO}_4$  with ammonium sulfate powder directly and the pH was adjusted to 6.5. The filtered sample (Nalgene Nunc, CAT #540887, MEMB 0.2 PES, Rochester, NY) was loaded to the 1<sup>st</sup> HIC column which was pre-equilibrated with 1.5 M  $(\text{NH}_4)_2\text{SO}_4$ , 20 mM potassium phosphate, 1 mM EDTA, pH 6.5 (Bed volume 150 ml, in XK-50, GE Healthcare, Piscataway, NJ). The ADA protein was eluted with an ammonium sulfate gradient and the purity profile of this elution was determined by SDS-PAGE and RP-HPLC. The ADA protein in the fractions of first HIC column was pooled and adjusted to 1 M  $(\text{NH}_4)_2\text{SO}_4$  and loaded directly to the second HIC column (Bed volume 150ml, XK-50, HIC Phenyl HP, Cat# 17-1082-01, Piscataway, NJ) which was pre-equilibrated with 1 M  $(\text{NH}_4)_2\text{SO}_4$ , 20 mM  $\text{KH}_2\text{PO}_4$ - $\text{K}_2\text{HPO}_4$ , 1 mM EDTA, pH 6.5. ADA was eluted with an ammonium sulfate gradient from 1 M to 300 mM in the 20 mM  $\text{KH}_2\text{PO}_4$ - $\text{K}_2\text{HPO}_4$ , 1 mM EDTA, pH 6.5. ADA purity of these fractions were analyzed by SDS-PAGE and RP-HPLC. The purified rbADA or rhADA was

further desalted and concentrated in a LabScale™ TFF systems (Membrane BioMax 5, Bedford, MA) against the storage buffer (for example, 100 mM sodium phosphate, 1 mM EDTA, pH 6.5).

#### EXAMPLE 7

##### PREPARATION OF PEGYLATED SER<sub>74</sub>-rbADA VIA URETHANE LINKAGE

SC-PEG (N-hydroxysuccinimidyl carbonate-activated polyethylene glycol, 0.084 mmol) is added to a solution of Ser<sub>74</sub>-rbADA (0.00027 mmol) in 3 mL of sodium phosphate buffer (0.1 M, pH 7.8) with gentle stirring. The solution is stirred at 30 °C for 30 minutes. A GPC column (Zorbax GF-450) is used to monitor PEG conjugation. At the end of the reaction (as evidenced by the absence of native enzyme), the mixture is diluted with 12 mL of formulation buffer (0.05 M sodium phosphate, 0.85% sodium chloride, pH 7.3) and diafiltered with a Centriprep concentrator (Amicon) to remove the unreacted PEG. Dialfiltration is continued as needed at 4 °C until no more free PEG is detected by mixing equal amount of filtrate and 0.1% PMA (polymethacrylic acid in 0.1 M HCl).

#### EXAMPLE 8

##### PREPARATION OF PEGYLATED SER<sub>74</sub>-rhADA VIA URETHANE LINKAGE

SC-PEG (0.084 mmol) is reacted with Ser<sub>74</sub>-rhADA (0.00027 mmol) using the same conditions as described in Example 7.

#### EXAMPLE 9

##### PREPARATION OF PEGYLATED SER<sub>74</sub>-rbADA VIA AMIDE LINKAGE

SS-PEG (N-hydroxysuccinimidyl succinate-activated polyethylene glycol, 0.084 mmol) is added to a solution of Ser<sub>74</sub>-rbADA (0.00027 mmol) in 3 mL of sodium phosphate buffer (0.1 M, pH 7.8) with gentle stirring. The solution is stirred at 30 °C for 30 minutes. A GPC column (Zorbax GF-450) is used to monitor PEG conjugation. At the end of the reaction (as evidenced by the absence of native enzyme), the mixture is diluted with 12 mL of formulation buffer (0.05 M sodium phosphate, 0.85% sodium chloride, pH 7.3) and diafiltered with a Centriprep

concentrator (Amicon) to remove the unreacted PEG. Dialfiltration is continued as needed at 4 °C until no more free PEG is detected by mixing equal amount of filtrate and 0.1% PMA (polymethacrylic acid in 0.1 M HCl).

#### EXAMPLE 10

#### PREPARATION OF PEGYLATED MUTEIN rhADA VIA AMIDE LINKAGE

SS-PEG (0.084 mmol) is reacted with mutein rhADA (0.00027 mmol) using the same conditions as described in Example 9.



We claim:

1. Use of an effective amount of an adenosine deaminase conjugate to treat a tumor or cancer of the prostate or ovaries in a patient;

wherein the adenosine deaminase is adenosine deaminase purified from a bovine source or is a recombinant bovine adenosine deaminase according to SEQ ID NO: 5 or SEQ ID NO: 1, or is a recombinant bovine adenosine deaminase according to SEQ ID NO: 1 or SEQ ID NO: 5, with an amino acid substitution selected from the group consisting of Gln in place of Lys<sub>198</sub>; Ala in place of Thr<sub>245</sub>; Arg in place of Gly<sub>351</sub> and combinations thereof; and

wherein the adenosine deaminase is conjugated to polyethylene glycol, and wherein the polyethylene glycol ranges in size from 4,000 to 45,000 Daltons.

2. The use of claim 1, wherein the tumor or cancer is malignant.

3. The use of claim 1 or 2, wherein the adenosine deaminase conjugate reduces tissue levels of adenosine or deoxyadenosine in said patient, and wherein growth or spread of the tumor is inhibited by reduced tissue levels of adenosine in said patient.

4. The use of any one of claims 1 to 3, wherein the tumor is a solid tumor.

5. The use of any one of claims 1 to 4, wherein the cancer is prostate cancer.

6. The use of any one of claims 1 to 4, wherein the cancer is ovarian cancer.

7. The use of any one of claims 1 to 4, wherein the tumor is a prostate tumor.

8. The use of any one of claims 1 to 4, wherein the tumor is an ovarian tumor.

9. The use of any one of claims 1 to 4, 7 or 8, wherein the adenosine deaminase conjugate is for use to maintain inhibition of the tumor.

10. The use of any one of claims 1 to 9, wherein the adenosine deaminase conjugate is for use for a time period ranging from 1 to 20 days.

11. The use of any one of claims 1 to 10, wherein the adenosine deaminase conjugate comprises two or more molecules of adenosine deaminase per non-antigenic polymer.
12. The use of any one of claims 1 to 11, wherein the adenosine deaminase conjugate comprises from 11 to 18 polyethylene glycol strands conjugated to epsilon amino groups of one or more Lys residues of the adenosine deaminase.
13. The use of claim 12, wherein the adenosine deaminase is conjugated to the polyethylene glycol strands via a urethane linkage.
14. The use of any one of claims 1 to 13, wherein the adenosine deaminase conjugate is for use by a route selected from the group consisting of subcutaneous, intravenous, intramuscular, intrathecal, intraperitoneal, inhalation and transurethral.
15. The use of any one of claims 1 to 14, wherein the recombinant bovine adenosine deaminase comprising SEQ ID NO: 5 comprises a Cys74 that is capped to prevent oxidation in an aqueous medium.
16. The use of any one of claims 1 to 11, wherein the polyethylene glycol is covalently attached to an epsilon amino group of a Lys of the adenosine deaminase.
17. The use of any one of claims 1 to 16, wherein the recombinant adenosine deaminase is recombinant adenosine deaminase comprising SEQ ID NO: 1.
18. The use of any one of claims 1 to 16, wherein the recombinant adenosine deaminase is recombinant adenosine deaminase comprising SEQ ID NO: 5.
19. The use of any one of claims 1 to 18, wherein the polyethylene glycol is about 5,000 Daltons.
20. The use of any one of claims 1 to 19, wherein the adenosine deaminase conjugate is ADAGEN<sup>®</sup>.