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(71) Demandeur/Applicant:
YEDA RESEARCH AND DEVELOPMENT CO. LTD, IL
(72) Inventeurs/Inventors:
SHECHTER, YORAM, IL;
FRIDKIN, MATITYAHU, IL;
TSUBERY, HAIM, IL
(74) Agent: BORDEN LADNER GERVAIS LLP

(54) Titre : **MEDICAMENTS PEGYLES REVERSIBLES**
(54) Title: **REVERSIBLE PEGYLATED DRUGS**

(57) **Abrégé/Abstract:**

Reversible pegylated drugs are provided by derivatization of free functional groups of the drug selected from amino, hydroxyl, mercapto, phosphate and/or carboxyl with groups sensitive to mild basic conditions such as 9-fluorenylmethoxycarbonyl (Fmoc) or 2-sulfo-9-fluorenylmethoxycarbonyl (FMS), to which group a PEG moiety is attached. In these pegylated drugs, the PEG moiety and the drug residue are not linked directly to each other, but rather both residues are linked to different positions of the scaffold Fmoc or FMS structure that is highly sensitive to bases and is removable under physiological conditions. The drugs are preferably drugs containing an amino group, most preferably peptides and proteins of low or medium molecular weight.



Abstract

Reversible pegylated drugs are provided by derivatization of free functional groups of the drug selected from amino, hydroxyl, mercapto, phosphate and/or carboxyl with groups sensitive to mild basic conditions such as 9-fluorenylmethoxycarbonyl (Fmoc) or 2-sulfo-9-fluorenylmethoxycarbonyl (FMS), to which group a PEG moiety is attached. In these pegylated drugs, the PEG moiety and the drug residue are not linked directly to each other, but rather both residues are linked to different positions of the scaffold Fmoc or FMS structure that is highly sensitive to bases and is removable under physiological conditions. The drugs are preferably drugs containing an amino group, most preferably peptides and proteins of low or medium molecular weight.

REVERSIBLE PEGYLATED DRUGS

This application is a divisional application of Canadian Patent Application No. 2,763,817, filed January 10, 2012, which is a divisional of Canadian Patent Application No. 2,521,784, filed April 8, 2004.

FIELD OF THE INVENTION

5 The present invention relates to reversible pegylation of drugs and to pegylated drugs that are slowly converted to the drugs in physiological conditions.

Abbreviations: ANP, atrial natriuretic peptide; t-Boc, tert-butyloxycarbonyl; BSA, bovine serum albumin; DCC, N,N'-dicyclohexylcarbodiimide; DCU, dicyclohexyl-
 10 lurea; DMF, dimethylformamide; DTNB, 5,5-dithiobis(2-nitrobenzoic acid); ESMS, electrospray ionization mass spectra; Fmoc, 9-fluorenylmethoxycarbonyl; Fmoc-OSu, Fmoc-N-hydroxysuccinimide ester; FMS, 2-sulfo-9-fluorenylmethoxycarbonyl; GSH, reduced glutathione; hGH, human growth hormone; HOSu, N-hydroxy-succinimide; HPLC, high-performance liquid chromatography;
 15 IFN- α 2, human interferon- α 2; ifnar2-EC, extracellular part of IFN- α 2 receptor; MAL-FMS-NHS, N-[2-(maleimido-propionyl amino)-7-sulfo-fluoren-9-yl-methoxy-carbonyloxy]succinimide (*Precursor 8*); MAL-FMS-OSu, MAL-FMS-NHS; NHS, N-hydroxy-succinimide; PBS, phosphate-buffered saline; PEG, polyethylene glycol; PEG₅₀₀₀, 5,000 Da-PEG; PEG₄₀ or PEG₄₀₀₀₀, 40,000 Da-branched PEG;
 20 PEG₄₀-SH, a 40 kDa-branched PEG containing a sulfhydryl moiety; PEG₄₀-OSu, PEG₄₀-N-hydroxysuccinimide ester; SC, subcutaneous; TFA, trifluoroacetic acid; TNBS, 2,4,6-trinitrobenzenesulfonic acid.

BACKGROUND OF THE INVENTION

25 Most peptide and protein drugs are short-lived and have often a short circulatory half-life *in vivo*. This is particularly valid for nonglycosylated proteins of a molecular mass less than 50 kDa. The short lifetime of proteins *in vivo* is attributed to several factors, including glomerular filtration in the kidney and

proteolysis. Considering that peptide and protein drugs are not absorbed orally, prolonged maintenance of therapeutically active drugs in the circulation is a desirable feature of obvious clinical importance. Proteins with molecular masses above ~60 kDa largely avoid glomerular filtration and are not, for the mainpart, filtered in the kidney. Therefore they remain in the circulation longer than smaller proteins.

An attractive strategy for improving clinical properties of small protein drugs has come to be known as PEGylation (or pegylation, as used hereinafter). By this strategy several hydrophilic chains of polyethylene glycol (PEG) are covalently linked to the protein in order to increase its effective molecular mass. Important clinical advantages are gained by pegylation. For example, life-time *in vivo* can be prolonged in some instances from minutes to hours, owing to the steric interference that protects conjugates from proteolysis *in vivo* and the increase in molecular mass, which precludes filtration by the kidney. Protein pegylation also decreases immunogenicity, presumably by protecting conjugates from being recognized as foreign antigens by the immune system.

In spite of the profound advantages often gained by pegylating therapeutic proteins, this technology suffers from a principal drawback. On the one hand, covalently attaching PEG chains to proteins prolongs their lifetime *in vivo*, protecting the conjugates from proteolysis and shielding them from the immune system. On the other hand, the steric interference of the PEG chains often leads to a drastic loss or even abolish the biological and the pharmacological potencies of the proteins in the conjugates (Fuertges and Abuchowski, 1990; Katre, 1993; Bailon and Berthold, 1998; Nucci et al., 1991; Delgado et al., 1992; Fung et al., 1997; Reddy, 2000; Veronese, 2001). In principle, this deficiency can be overcome by introducing the PEG chains via a chemical bond that is sensitive to hydrolysis, or can be cleaved enzymatically by serum proteases or esterases. Clearly, a consistent rate of hydrolysis is crucial. A prerequisite condition is therefore that the hydrolysis of the PEG chains from the conjugate is to take place at a slow rate, and in a homogenous fashion *in vivo*.

It would be highly desirable to design PEG derivatives of proteins or peptides or small drug molecules from which PEG can be released by hydrolysis. An appropriate reversible PEG conjugate would have to be hydrolyzed slowly and spontaneously in physiological conditions and would permit time-dependent
5 reactivation of inactive pegylated proteins and peptides.

Several methods for reversible pegylation were proposed (Greenwald et al., 1999, 2000; Lee et al., 2001; Garman and Kalindjian, 1987; Zalipsky et al., 1999). They suffer, however, from major potential drawbacks. For example, reliance on enzymatic detachment as a rate-determining step (Greenwald et al., 1999, 2000; Lee
10 et al., 2001) of PEGs from conjugates by serum proteases and/or esterases might not yield desirable pharmacokinetic profiles *in situ*. Moreover, it is dependent on enzymes availability. Disulfide-bonded conjugate is not to be cleaved in the non-reducing environment of the body fluids (Zalipsky et al., 1999). A reversibly
15 SH functions may result in complex undesired cross-linking (Garman and Kalindjian, 1987). It would be very desirable to design a version of reversible pegylation that would overcome these deficiencies.

International PCT Publication No. WO 98/05361 of the present applicants describes a novel conceptual approach for prolonging the half-life of drugs by
20 derivatizing a drug having at least one free amino, carboxyl, hydroxyl and/or mercapto groups with a moiety that is highly sensitive to bases and is removable under mild basic conditions. The prodrug obtained is inactive but undergoes transformation into the active drug under physiological conditions in the body. Examples of said moieties are the radicals 9-fluorenylmethoxycarbonyl (Fmoc) and
25 2-sulfo-9-fluorenylmethoxycarbonyl (FMS). According to this concept, Fmoc and FMS derivatives of peptidic drugs such as insulin and human growth hormone as well as of non-peptidic drugs such as propranolol, cephalixin and piperacillin have been described in said WO 98/05361. Later on, FMS derivatives of cytokines have been disclosed in WO 02/36067, and FMS derivatives of enkephalin, doxorubicin,

amphotericin B, gentamicin and gonadotropin releasing hormone (GnRH) were disclosed in WO 02/7859.

US Patent No. 6,433,135 discloses a pegylated derivative of an analogue of luteinizing hormone releasing hormone (LHRH or GnRH) in which the PEG moiety is covalently bound to a serine residue of said LHRH analogue. In the process of preparation of said PEG-LHRH analogue by solid phase peptide synthesis, a pegylated serine residue such as Fmoc-Ser(PEG)-OH or tBoc-Ser(PEG)-OH is introduced into the LHRH analogue, and the produced PEG-LHRH analogue is recovered (without the protective group Fmoc or t-Boc).

JP Patent Application JP 3148298 describes PEG-peptide conjugates, e.g., PEG-GnRH conjugate, obtained by reacting the guanidino group of an arginine residue with PEG, while protecting the amino groups present in the molecules.

Citation of any document herein is not intended as an admission that such document is pertinent prior art, or considered material to the patentability of any claim of the present application. Any statement as to content or a date of any document is based on the information available to applicants at the time of filing and does not constitute an admission as to the correctness of such a statement.

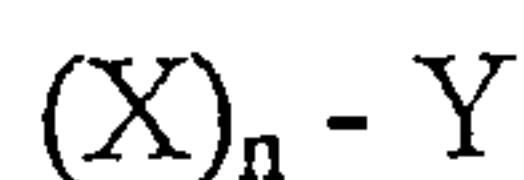
SUMMARY OF THE INVENTION

It has now been found, in accordance with the present invention, that by combination of the protein-pegylation technology with the technology of derivatization with Fmoc or FMS or similar moieties removable under mild basic conditions, major deficiencies of the protein-pegylation technology, mainly the loss of biological and pharmacological potencies in the PEG conjugates *in vivo*, may be overcome.

It is thus one object of the present invention to design PEG-protein conjugates from which PEG can be released by hydrolysis under physiological conditions in the body.

It is another object of the present invention to provide reversible PEG-protein conjugates that are inactive when administered and permit time-dependent reactivation of the inactivated pegylated protein under physiological conditions in the body.

5 The present invention thus relates, in one aspect, to a compound of the formula:



wherein

10 Y is a moiety of a drug bearing at least one functional group selected from free amino, carboxyl, phosphate, hydroxyl and/or mercapto, and

X is a radical that is highly sensitive to bases and is removable under mild basic conditions, said radical carrying a linear or branched PEG moiety;

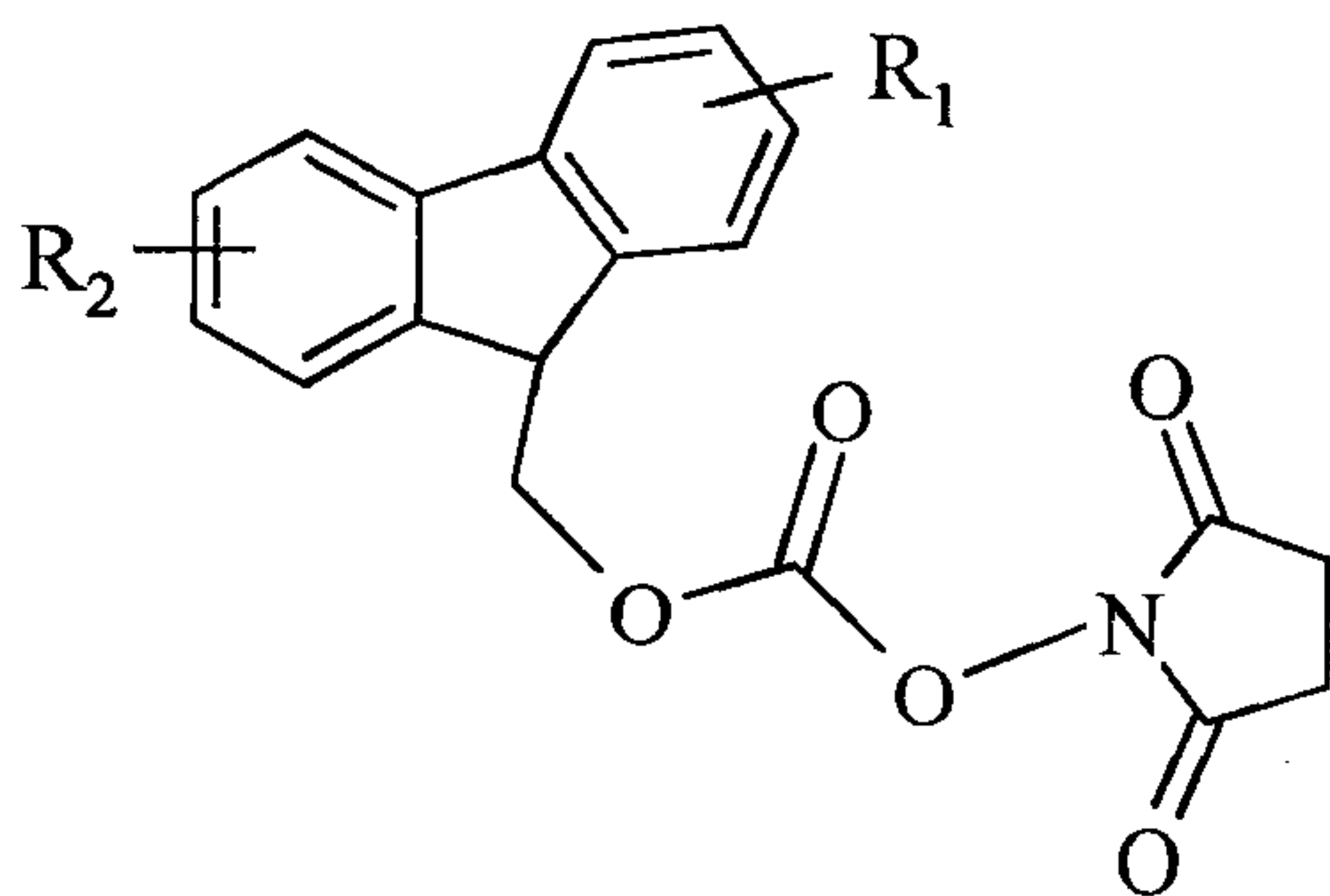
n is an integer of at least one, and pharmaceutically acceptable salts thereof.

15 The prodrug obtained is inactive but undergoes transformation into the active drug Y under physiological conditions in the body.

In preferred embodiments of the invention, the radical X is Fmoc or 2-sulfo-Fmoc (herein "FMS"), Y is a peptide or protein drug linked to Y through an amino group, n is 1 or 2, and the linear or branched PEG moiety has a molecular weight of 5,000-40,000 Da.

20 In another aspect, the present invention provides novel methods and intermediates and precursors for the preparation of the conjugates of the invention.

There is described herein an intermediate compound of the formula:



wherein:

R₁ is a radical of the formula:



wherein

R_2 is H or $-SO_3H$ at position 2 of the fluorene ring;

B is maleimido, $-S-CO-CH_3$ or a PEG moiety;

R_5 is $-NH-$, $-S-$, $-CO-$, $-COO-$, $-CH_2-$, $-SO_2-$, $-SO_3-$, $-PO_2-$, or $-PO_3-$; and

R_6 is a bond or a radical by which the maleimido, $-S-CO-CH_3$ or PEG moiety is attached to R_5 .

In a further aspect, the present invention provides pharmaceutical compositions comprising a pharmaceutically acceptable carrier and a prodrug of the invention.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 shows the stability of the maleimide functional moiety in MAL-FMS-NHS in aqueous solutions having different pH values. MAL-FMS-NHS (1 mM) was incubated at room temperature in H_2O (pH 6.0), in 0.007 M acetic acid

(pH~4.0), in 0.1 M phosphate buffer (pH 7.4), and in 0.1 M NaHCO₃ (pH 8.5). At the indicated time points, aliquots were allowed to react with a slight excess of GSH (15 min at pH 7.2) and the concentration of unreacted GSH was determined with 5,5-dithiobis(2-nitrobenzoic acid) (DTNB).

5 Fig. 2 shows the degree of incorporation of MAL-FMS-NHS at pH 7.2 into α -lactalbumin (α -LA) as a function of the amount of added reagent. To samples of α -LA (1.0 ml of 1mg/ml in 0.1M phosphate buffer, pH 7.2), MAL-FMS-NHS was added at concentrations ranging from 1 equivalent up to 14 molar equivalents of MAL-FMS-NHS. For each treatment the amount incorporated into the protein was
10 determined by the absorbance at 280 nm, after dialysis, and by quantitating the amount of unmodified amino-side chain moieties with trinitrobenzene sulfonic acid. (TNBS).

 Figs. 3A-3B show the time course of reactivation of (PEG₅₀₀₀-Fmoc)₂-gentamicin and (PEG₅₀₀₀-Fmoc)₁-gentamicin conjugates, respectively. After
15 incubation at pH 8.5, 37°C, aliquots were withdrawn at the indicated time points and analyzed for their potency to arrest *E. coli* replication. The IC₅₀ for each aliquot was determined. Native gentamicin inhibited *E. coli* replication with IC₅₀ value = 0.22±0.02 μ M.

 Figs. 4A-4B show progressive modification of the amino acid moieties of
20 human insulin with PEG₅₀₀₀-Fmoc-OSu and loss of biological potency as a function of PEG₅₀₀₀-Fmoc incorporated into insulin, respectively. (4A) Insulin (17.24 nmoles in 0.2 ml 0.01 M NaHCO₃) reacted with increasing concentrations of PEG₅₀₀₀-Fmoc-OSu at a molar excess over the protein as indicated in the figure for 2 hours at 25°C. The number of free amino groups that remained unmodified were
25 quantitated with TNBS. (4B) Aliquots containing 0.4, 0.7, 1.1, 1.5 and 2.2 moles PEG₅₀₀₀-Fmoc covalently attached per mole insulin, were assayed for their lipogenic potency in rat adipocytes. Under the assay conditions, human insulin stimulates lipogenesis, 4-6 times above basal levels with ED₅₀ value of 0.2±0.02 ng/ml. An insulin derivative exhibiting ED₅₀ of 2.0±0.2 ng/ml in this assay is
30 considered as having 10% the lipogenic potency of native insulin.

Fig. 5 shows the rate of reactivation of PEG₅₀₀₀-Fmoc-insulin conjugates upon incubation at pH 8.5, 37°C. PEG₅₀₀₀-Fmoc-insulin conjugates containing one and two moles of PEG₅₀₀₀-Fmoc/mole insulin were incubated at a concentration of 0.172 μM in 0.1 M NaHCO₃-0.5% bovine serum albumin and 1 mM NaN₃ at 5 37°C. At the indicated time points aliquots were analyzed (in several concentrations for each aliquot) for their lipogenic potencies in rat adipocytes.

Fig. 6 shows prolonged glucose-lowering effect after a single subcutaneous (SC) administration of (PEG₅₀₀₀-Fmoc)₁-insulin in mice. Mice received SC, either native insulin (Zn²⁺-free, 1.72 nmole/mouse in 0.2 ml PBS buffer) or (PEG₅₀₀₀-Fmoc)₁-insulin (17.2 nmole/mouse, in 0.2 ml PBS buffer). Blood glucose levels were determined at the indicated time points. Each point is the arithmetic mean±SEM of blood glucose of five mice. 10

Fig. 7 shows glucose-lowering pattern in mice following single intraperitoneal (IP) administration of (PEG₅₀₀₀-Fmoc)₁-insulin. Groups of mice received IP either insulin (Zn²⁺-free, 0.345 nmol/mouse, in 0.2 ml PBS buffer) or (PEG₅₀₀₀-Fmoc)₁-insulin (3.45 nmoles/mouse, in 0.2 ml PBS buffer). Blood glucose levels were determined at the indicated time points. Each point in the figure is the arithmetic mean±SEM of five mice. 15

Fig. 8 shows the rate of release of exendin-4 from PEG₄₀-FMS-exendin-4 conjugate, upon incubation at pH 8.5, 37°C. At the indicated time points, aliquots (50 μl) were loaded on HPLC, and ran under conditions resolving well exendin-4 from the conjugate. Results are expressed as percent of maximal peak area of released exendin-4, as a function of time. Exendin-4 (50 μg) was assigned at 100% peak area. 20

Fig. 9 shows the rate of hydrolysis of PEG-FMS conjugates, upon incubation at pH 8.5, 37°C. Solutions of PEG₅₀₀₀-FMS-exendin-4 (circles) and PEG₅₀₀₀-FMS-4-nitro-phenethyl amine (squares) were incubated in PBS at pH 8.5, 37°C. At the indicated time points, aliquots (50 μl) were analyzed using HPLC on a RP-4 25

column. Results are expressed as percent of the maximal peak area of released exendin-4 and 4-nitrophenethylamine, as a function of time.

Figs. 10A-10B show glucose-lowering patterns of native exendin-4 and PEG₄₀₀₀₀-FMS-exendin-4 following a single subcutaneous administration to CD1-mice. (10A) CD1-mice were SC-administered with either native exendin-4 (10 μg/mouse) or with PEG₄₀-FMS-exendin-4 (10 μg/mouse of exendin-4 equiv). At the indicated time points,, circulating glucose levels were determined. Each experimental group consisted of five mice. Data are presented as means±SE. (10B) Three groups of CD1 mice (n=6 per group) underwent one subcutaneous administration of saline, native exendin-4 (4 μg/mouse) or PEG₄₀₀₀₀-FMS-exendin-4 (4μg peptide/mouse). Circulating glucose levels were then monitored. Results are expressed as percent decrease in plasma glucose concentration in the groups treated with exendin-4 or PEG₄₀₀₀₀-FMS-exendin-4 relative to that found in the saline-treated group measured at the same time-point during the day.

Figs. 11A-11C show release of active IFNα2 upon incubation of PEG₄₀-FMS-IFNα2 at pH 8.5, 37°C. PEG₄₀-FMS-IFNα2 (0.3 mg protein/ml) was incubated in 0.1 M phosphate buffer with 2 mM NaN₃ and 6 mg/ml BSA (pH 8.5, 37°C). At the indicated time points, aliquots were withdrawn. (11A) Analysis of IFNα2 discharge from the conjugate by SDS-PAGE; the amounts of IFNα2 discharge were quantified relative to an IFNα2 reference of known concentration and intensity (the time increments and the percentages are indicated); (11B) Aliquots withdrawn at the indicated time points were analyzed for their Ifnar2 binding capacity on BIAcore; (11C) Fitted BIAcore profile of native IFNα2 discharge from PEG₄₀-FMS-IFNα2.

Fig. 12 shows the results of SC administration of native IFNα2 and PEG₄₀-FMS-IFNα2. Rats were SC injected with the indicated concentrations of native IFNα2 (100 μg/rat) or the PEG₄₀-FMS-IFNα2 conjugate (12, 60, 120 μg/rat) (0.2 ml/rat, dissolved in PBS). Blood aliquots were withdrawn at the indicated time

points. Circulating antiviral activities in the aliquots were determined in human WISH cells with 3-fold serial dilutions of each aliquot.

Fig. 13 shows the result of intravenous administration of PEG₄₀-FMS-IFN α 2 to rats. Rats were intravenously injected with the indicated concentrations of native IFN α 2 (30 μ g/rat) or the PEG₄₀-FMS-IFN α 2 conjugate (30 μ g/rat) (0.2 ml/rat, dissolved in PBS). Blood aliquots were withdrawn at the indicated time points. Circulating antiviral activities in the aliquots were determined in human WISH cells with 3-fold serial dilutions of each aliquot.

Figs. 14A-14B show experimental vs. simulated behavior of IFN α 2: (14A) following SC administration, with initial concentrations of 60 nM and 1.5 nM of PEG₄₀-FMS-IFN α 2 and native IFN α 2, respectively; (14B) following intravenous administration to rats with initial concentrations of 20 nM of PEG₄₀-FMS-IFN α 2, 1.5 nM of native IFN α 2 in the SC volume and no conjugate in circulation. The inserts are the experimental curves.

Fig. 15 shows dose-response of PYY₃₋₃₆ in food intake in mice. Male C57BL6J mice (10 per group), were deprived of food for 24 h. At time 23 h, the mice received a SC injection of either saline or the indicated doses of PYY₃₋₃₆. At time 24 h the mice were allowed to consume an excess of pre-weighted chow for 2 h. Drinking water was provided at all times. The amount of food consumed per 10 mice during 2 h is shown as a function of PYY₃₋₃₆ dose.

Fig. 16 shows time-dependent reduction in food intake in mice by PYY₃₋₃₆. Male C57BL6J mice (10 per group) were deprived of food as described in Fig. 15 and PYY₃₋₃₆ (5 nmol/mouse) was administered at the indicated times prior to start of the re-feeding period. Results are average of four identical experiments.

Fig. 17 shows the effects of irreversible pegylation on the biological activity of PYY₃₋₃₆. Native PYY₃₋₃₆ was allowed to react with PEG₄₀-OSu. Groups of 10 mice were injected SC with saline, PYY₃₋₃₆, or PEG₄₀- PYY₃₋₃₆ (5 nmol/mouse) at 1 h prior to start of the re-feeding period. Results are the average of two identical experiments.

Fig. 18 shows that PEG₄₀-FMS is linked to the α -amino group of PYY₃₋₃₆. PEG₄₀-FMS-PYY₃₋₃₆ (100 μ g) was acetylated by a 500 molar excess of acetic anhydride at pH 7.0, dialyzed, and incubated for 3 days at pH 8.5, 37°C to quantitatively remove the PEG₄₀-FMS moiety. The resulting acetylated PYY₃₋₃₆ was then subjected to three cycles of N-terminal protein sequence analysis. The sequence obtained was ile-(N ϵ -acetyl)lys-pro. Sequence analysis of the native peptide yielded ile, lys, and pro on cycles 1, 2, 3, respectively (not shown).

Fig. 19 shows the kinetics of PYY₃₋₃₆ release from PEG₄₀-FMS-PYY₃₋₃₆. PEG₄₀-FMS-PYY₃₋₃₆ (750 μ M in 0.1 M phosphate buffer pH 8.5, 2 mM NaN₃), was incubated at 37°C. Aliquots (100 μ l) were withdrawn at the indicated times and free PYY₃₋₃₆ was measured by HPLC. The cumulative amount of PYY₃₋₃₆ released is shown as a function of time. The amount of PYY₃₋₃₆ in the initial conjugate was determined by acid hydrolysis of a 20 μ l aliquot, followed by amino acid analysis.

Fig. 20 shows the rate of PEG₄₀-FMS-PYY₃₋₃₆ hydrolysis in normal mouse serum. PEG₄₀-FMS-PYY₃₋₃₆ (0.5 μ M) in normal mouse serum was incubated at 37°C. Aliquots were withdrawn at the indicated times and the amount of 2-PEG₄₀-9-sulfo-fulvene released from PEG₄₀-FMS-PYY₃₋₃₆ was determined by HPLC and taken for calculating the rate of PEG₄₀-FMS-PYY₃₋₃₆ hydrolysis. The insert shows that PYY₃₋₃₆ degrades rapidly in normal mouse serum at 37°C. PYY₃₋₃₆ (50 nM) in normal mouse serum was incubated at 37°C. At the indicated times, aliquots (0.1 ml) were removed, de-proteinated by 3 volumes of ethanol and the quantity of PYY₃₋₃₆ was determined in the supernatants by HPLC.

Fig. 21 shows that PEG₄₀-FMS-PYY₃₋₃₆ elicits prolonged satiety. The protocol described in Fig. 15 was repeated, except that the mice received SC either saline or PEG₄₀-FMS-PYY₃₋₃₆ (5 nmol/mouse) at the indicated times prior to re-feeding. Results are average of three identical experiments, normalized according to the saline control.

Fig. 22 shows the time course of reactivation of PEG₄₀₀₀₀-FMS-hGH upon incubation at pH 8.5, 37°C. PEG₄₀₀₀₀-FMS-hGH (1 mg/ml) was incubated in 0.1 M

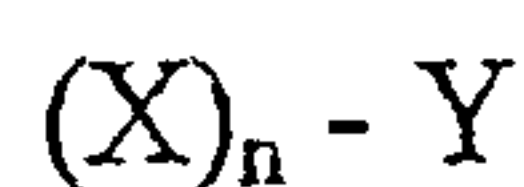
phosphate buffer, 0.6% BSA and -2 mM NaN₃ at 37°C. Aliquots were withdrawn at the indicated time points, and analyzed for their potencies to displace ¹²⁵I-hGH from enriched hGH-receptor preparation extracted from rabbit liver plasma membranes. Native hGH displaces ¹²⁵I-hGH in this assay half maximally at a concentration of 0.3±0.03 nM. An hGH derivative exhibiting half maximal displacement in this assay at a concentration of 3.0±0.3 nM is considered to have 10% of the native receptor's binding potency. The insert shows the rate of release of hGH from PEG₄₀-FMS-hGH upon incubation at pH 8.5, 37°C. PEG₄₀₀₀₀FMS-hGH (1 mg protein) was incubated as described above. At the indicated time points, 0.1 ml aliquots withdrawn and subjected to analytical HPLC analysis.

Fig. 23 shows the rate of hydrolysis of PEG-FMS conjugates, upon incubation at pH 8.5, 37°C. The concentration of PEG₄₀₀₀₀-FMS-exendin-4 and PEG₄₀₀₀₀-FMS-hGH was determined for each time point by HPLC. The linear plot obtained indicates that the rate of hydrolysis is of first order reaction. The half-life time of the conjugates was calculated from $t_{1/2} = \ln 2/k$, when k is the slope of the linear plot (h⁻¹).

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a new conceptual approach for reversible pegylation of drugs, particularly peptides and proteins of low or medium molecular weight, whereby the PEG moiety and the drug residue are not linked directly to each other, as in standard pegylation procedures, but rather both residues are linked to different positions of a scaffold structure that is highly sensitive to bases and is removable under physiological conditions.

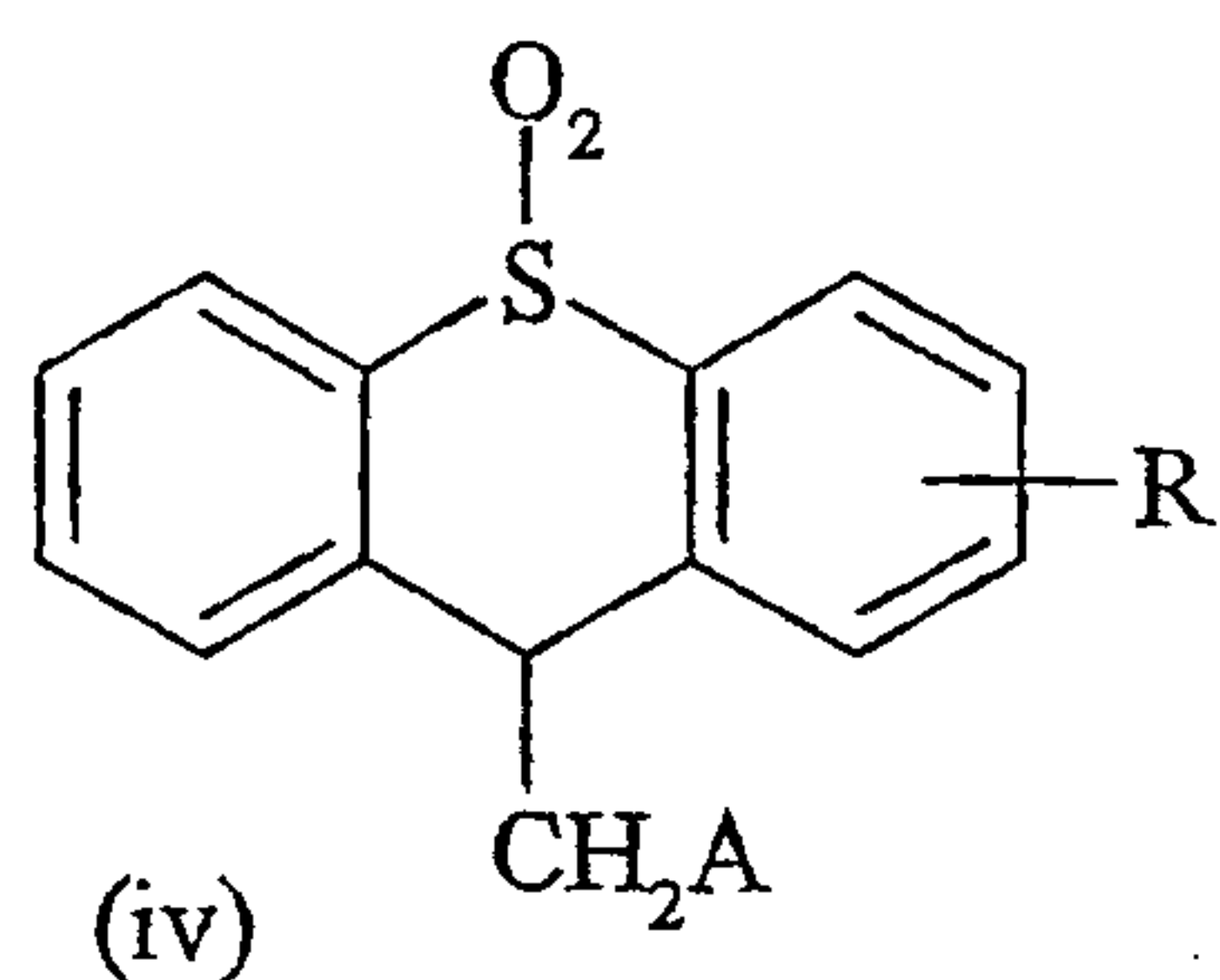
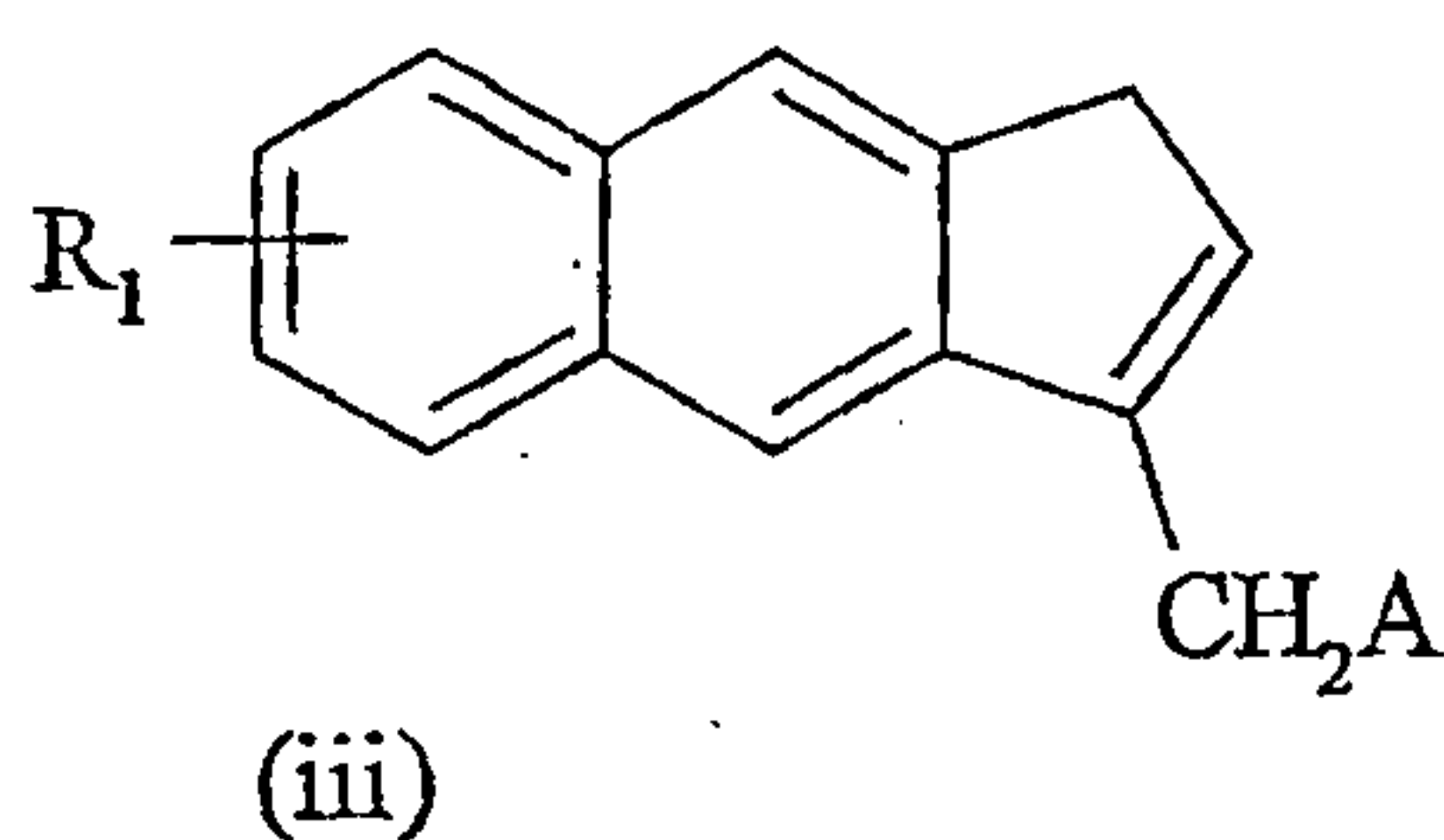
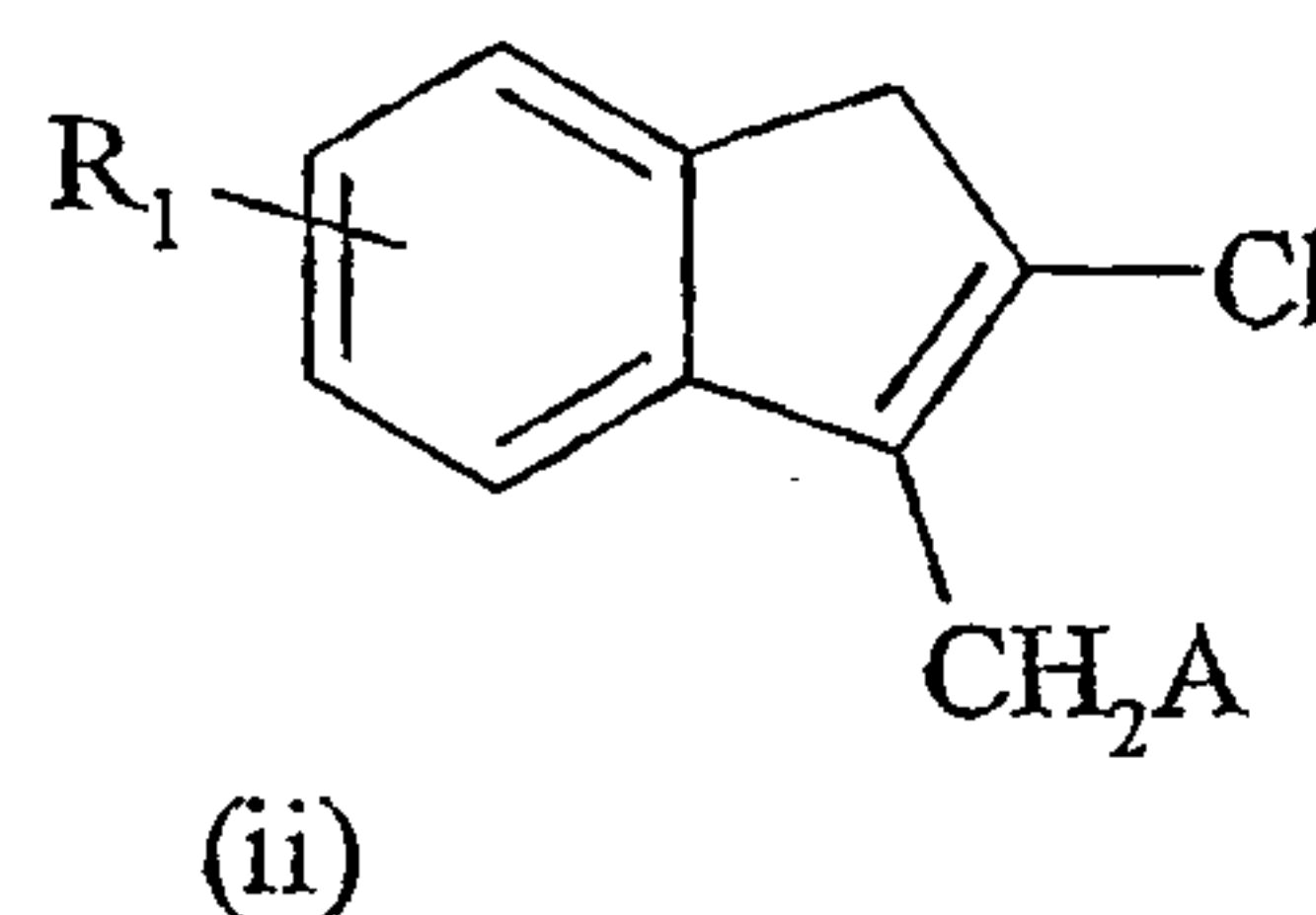
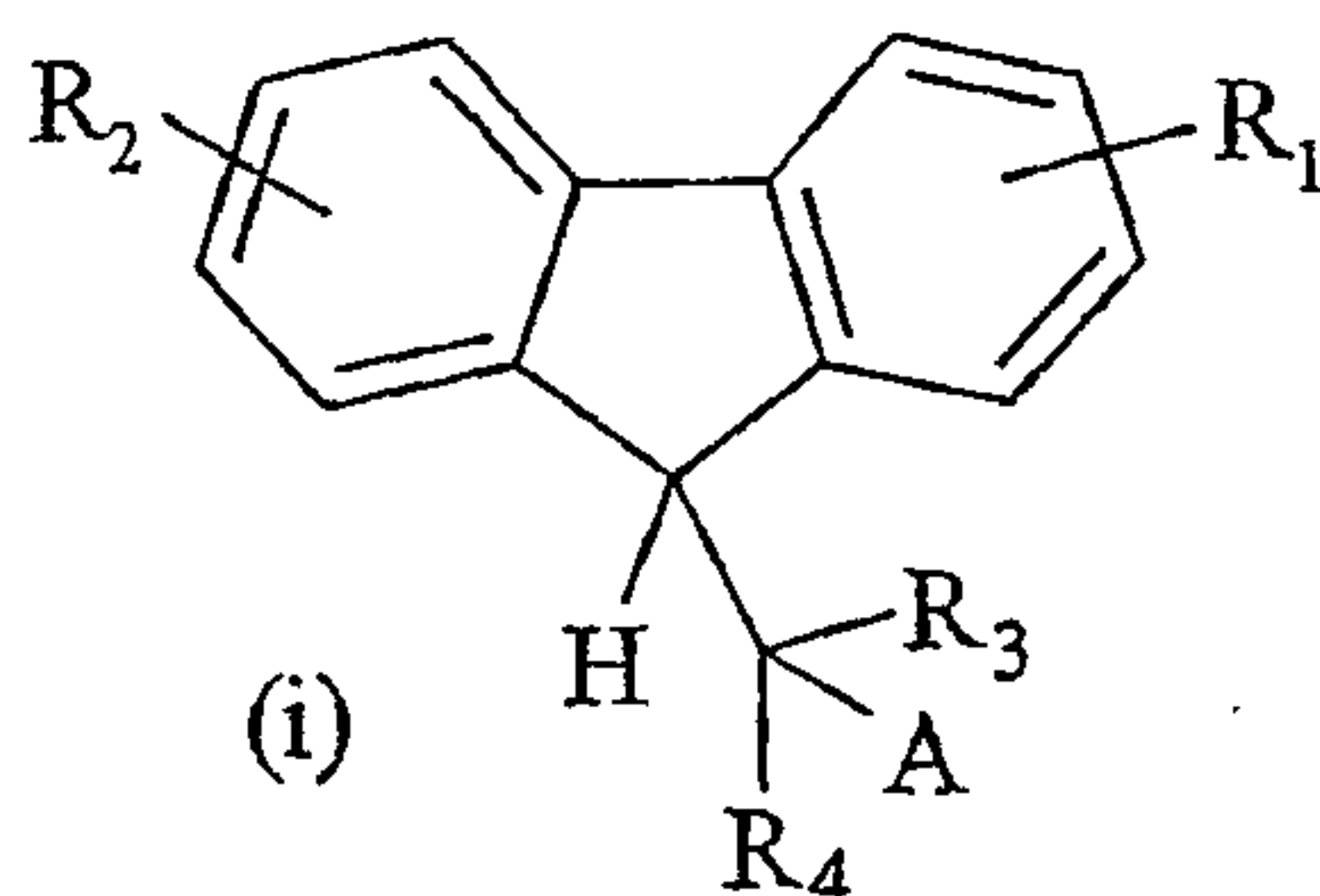
In one aspect, the present invention provides a compound of the formula:



wherein

Y is a moiety of a drug bearing at least one functional group selected from free amino, carboxyl, phosphate, hydroxyl and/or mercapto, and

X is a radical selected from the group of radicals consisting of the formulas (i) to (iv):



5

wherein:

R_1 is a radical containing a polyethylene glycol (PEG) moiety;

R_2 is selected from the group consisting of hydrogen, alkyl, alkoxy, alkoxyalkyl, aryl, alkaryl, aralkyl, halogen, nitro, $-SO_3H$, $-SO_2NHR$, amino, ammonium, carboxyl, PO_3H_2 , and OPO_3H_2 ;

R is selected from the group consisting of hydrogen, alkyl and aryl;

R_3 and R_4 , the same or different, are each selected from the group consisting of hydrogen, alkyl and aryl;

A is a covalent bond when the radical is linked to a carboxyl, phosphate or mercapto group of the drug Y , or A is $OCO-$ when the radical is linked to an amino or hydroxyl group of the drug Y ;

n is an integer of at least one,

and pharmaceutically acceptable salts thereof.

The terms "alkyl", "alkoxy", "alkoxyalkyl", "aryl", "alkaryl" and "aralkyl" in the definitions of R_1 , R_2 , R_3 and R_4 herein are used to denote alkyl radicals of 1-

8, preferably 1-4 carbon atoms, e.g. methyl, ethyl, propyl, isopropyl and butyl, and aryl radicals of 6-10 carbon atoms, e.g. phenyl and naphthyl. The term "halogen" includes bromo, fluoro, chloro and iodo.

In one preferred embodiment of the invention, X is a radical of the formula
 5 (i), more preferably a radical of formula (i) wherein R_2 , R_3 and R_4 are each hydrogen and A is $-OCO-$, namely the 9-fluorenylmethoxycarbonyl radical (hereinafter "Fmoc"), or most preferably, a radical of formula (i) wherein R_2 is $-SO_3H$ at position 2 of the fluorene ring, R_3 and R_4 are each hydrogen, and A is $-OCO-$, namely the 2-sulfo-9-fluorenylmethoxycarbonyl radical (hereinafter
 10 "FMS").

In another embodiment of the invention, the functional group is the radical (i), wherein R_2 , R_3 and R_4 are hydrogen and A is a covalent bond, i.e. the 9-fluorenylmethyl (Fm) group, which is applicable for reversible masking of free mercapto groups, of carboxylic functions of aspartic and glutamic acid moieties,
 15 and of C-terminal carboxyl functions of the cytokine molecules. The resulting 9-fluorenylmethyl esters (Fm-esters) generate the parent free carboxylic functions following a β -elimination reaction pathway upon mild basic treatment, and thus can be similarly employed for reversible masking of carboxylic functions of drugs. The Fmoc-group is of further potential similar use in the reversible protection of
 20 hydroxyl groups of tyrosine, serine and threonine.

The halogenated Fmoc radicals (i) wherein R_2 is halogen in the 2 or 7 position, preferably Cl or Br, the 2-chloro-1-indenylmethoxycarbonyl (CLIMOC) radical (ii), the 1-benzo[f]indenylmethoxycarbonyl urethane (BIMOC) radical (iii), the urethane sulfone radical (iv) and corresponding radicals (i) to (iv) wherein A is a
 25 covalent bond, can be used similarly to Fmoc and Fm for substitution of free amino, carboxyl, hydroxyl and mercapto functions of drugs, thus providing a wide range of sensitivity toward removal of such groups under basic, e.g. physiological, conditions. In fact, the above radicals (i) to (iv) belong to a general family of rare chemical entities that undergo hydrolysis at neutral or slightly alkaline pH and mild
 30 conditions, and can therefore be used for temporary reversible protection of α - and

ϵ -amino groups, for example in peptide synthesis, and can be removed from the amino function by a β -elimination reaction, under mild basic conditions.

According to the invention, a radical (i) to (iv), preferably Fmoc or FMS covalently linked to amino and/or hydroxyl moieties or Fm covalently linked to
5 carboxyl and/or mercapto moieties, undergoes hydrolysis (via β -elimination) back to the free amino, hydroxy, mercapto or carboxyl functions, under physiological conditions in the body fluid, namely at pH 7.4 and 37°C.

In one embodiment of the invention, R₁ is a radical of the formula:



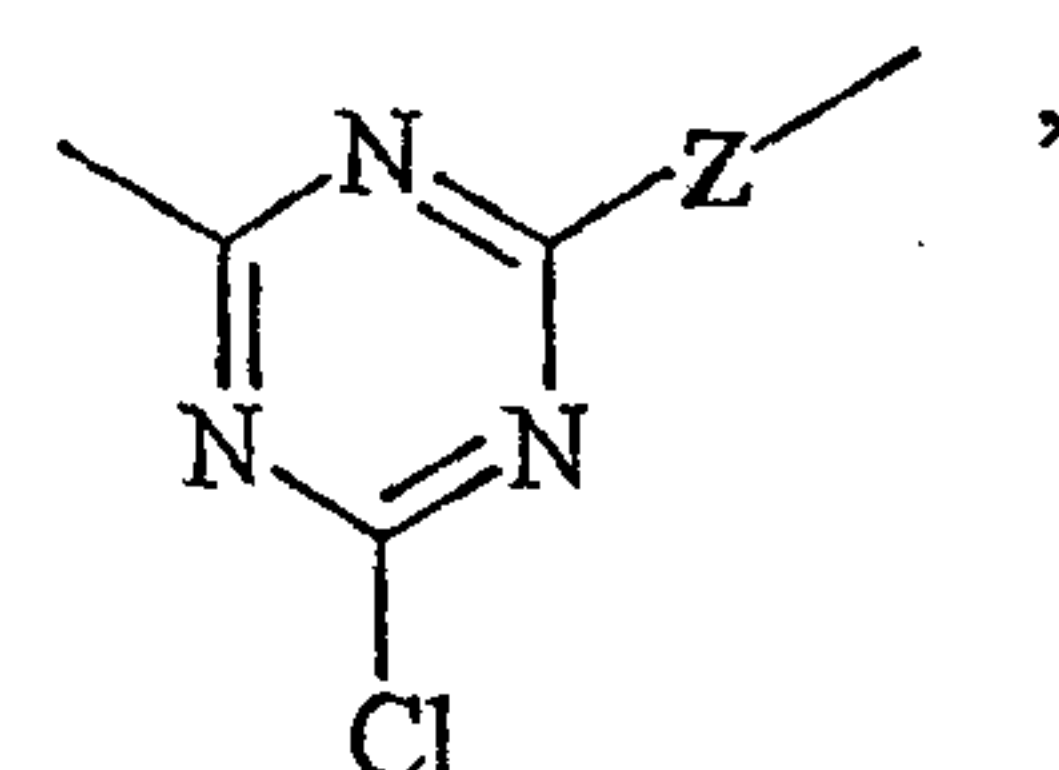
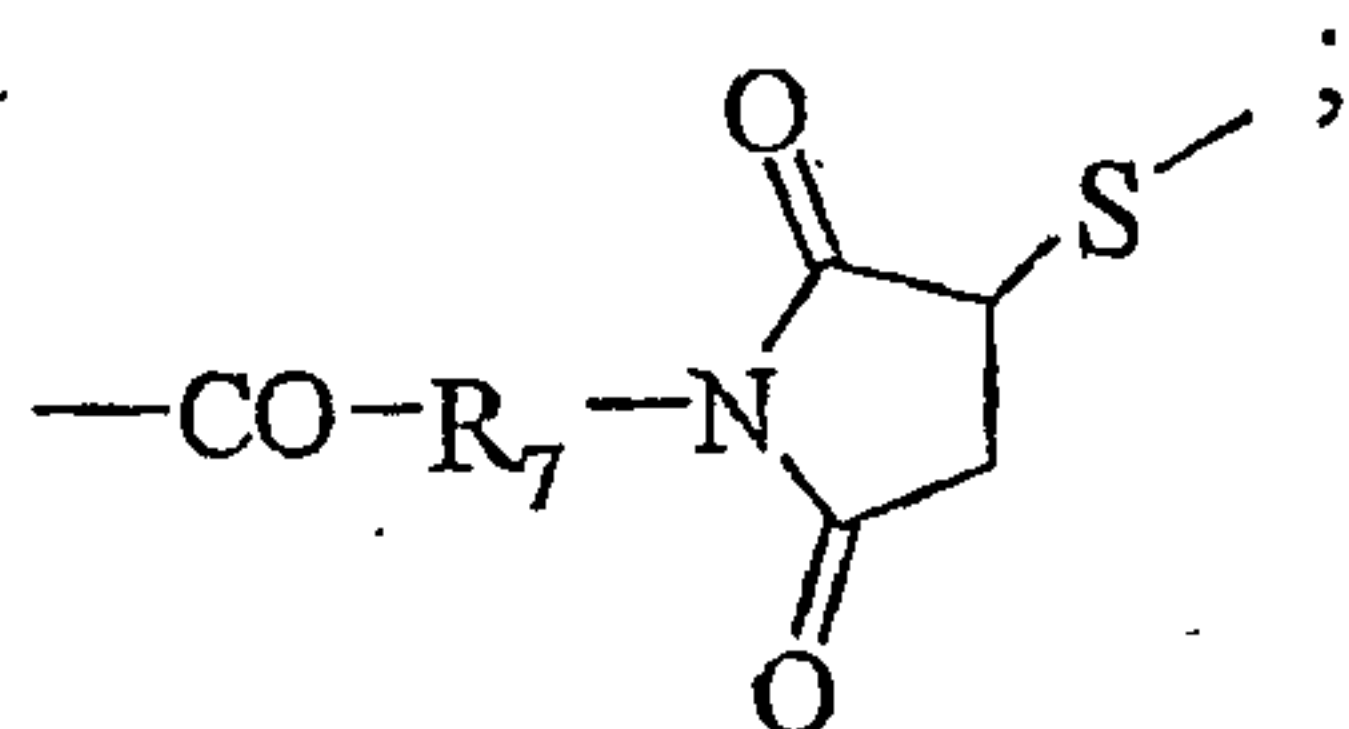
10 wherein

R₅ is selected from the group consisting of -NH-, -S-, -CO-, -COO-, -CH₂-, -SO₂-, -SO₃-, -PO₂-, and -PO₃-; and

R₆ is a bond or a radical by which the PEG moiety is covalently attached to R₅.

15 In a more preferred embodiment, R₅ is -NH-, and R₆ is selected from the group consisting of -CO-, -COO-, -CH₂-, -CH(CH₃)-, CO-NH-, -CS-NH-, -CO-CH₂-NH-CO-, -CO-CH(CH₃)-NH-CO-, -CO-CH₂-NH-CO-NH,

and

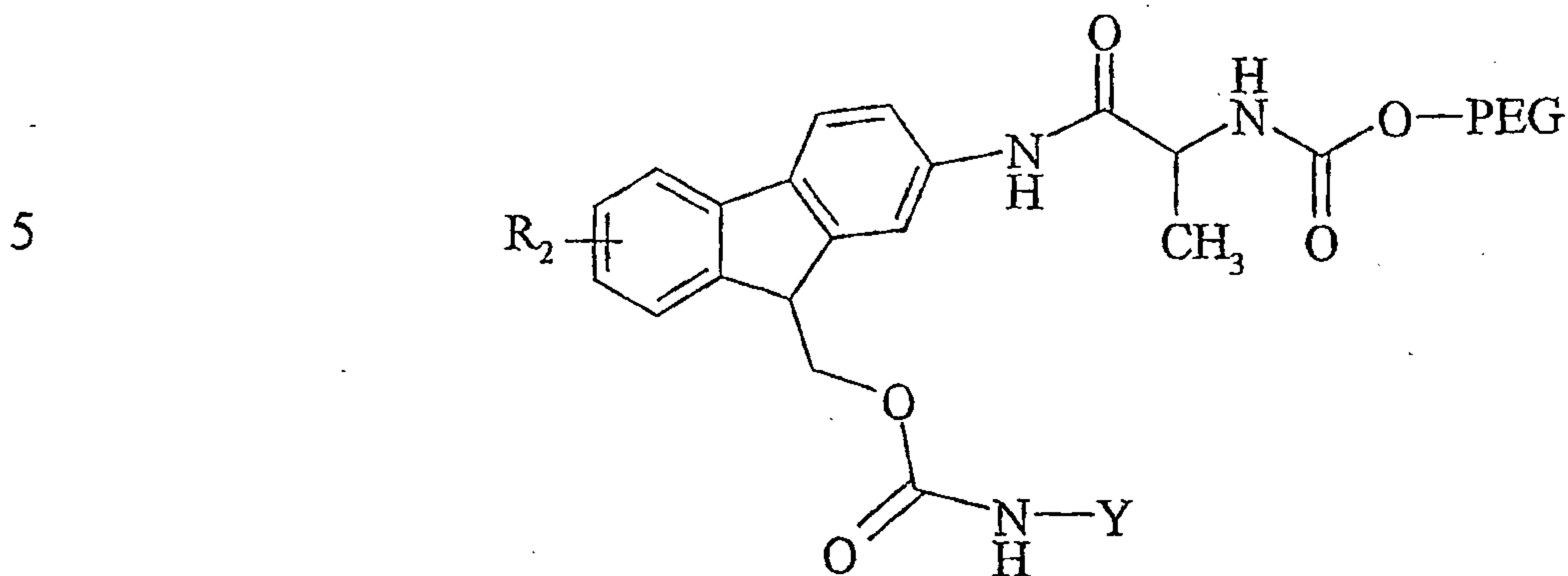


Z is O, S or NH; and

R₇ is selected from the group consisting of C1-C18 straight or branched alkylene, phenylene, an oxyalkylene radical having 3-18 carbon atoms in the
25 backbone, a residue of a peptide containing 2-10 amino acid residues, and a residue of a saccharide containing 1-10 monosaccharide residues.

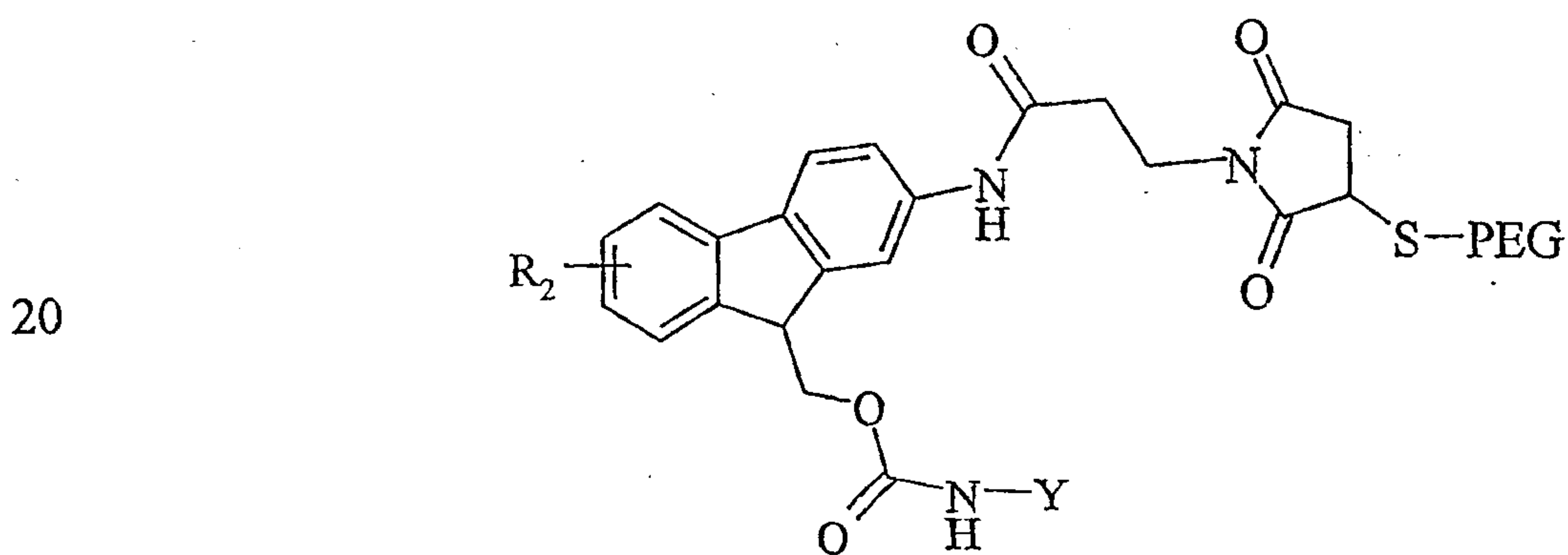
In the 4-chloro-6-Z-triazin-2-yl radical above, the 6-Z- group is linked to the PEG moiety while the 2 position is linked to R₅, which is -NH- in this case. In the -CO-R₇-succinimido radical above, the thio -S- group at position 3 is linked to the
30 PEG moiety while the -CO- is linked to R₅, which is -NH- in this case.

In one preferred embodiment, the pegylated drug compound of the invention is a conjugate of the formula:



10 wherein R_2 is H or $-SO_3H$ at position 2 of the fluorene ring, and Y is preferably a peptide or protein drug. When R_2 is H, a herein designated PEG-Fmoc-drug Y conjugate is obtained. In a most preferred embodiment, R_2 is $-SO_3H$ at position 2 of the fluorene ring, and a herein designated PEG-FMS-drug Y conjugate is obtained.

15 In a more preferred embodiment, the pegylated drug of the invention is a compound of the formula:



wherein R_2 is H or $-SO_3H$.

25 In a most preferred embodiment, the pegylated drug of the invention is a compound of the formula above, wherein R_2 is $-SO_3H$ at position 2 of the fluorene ring, and the PEG moiety is a 40 kDa branched PEG. These conjugates are herein identified as $(PEG_{40}\text{-FMS})_n\text{-peptide/protein}$, wherein n is 1 to 3, preferably 1 or 2, most preferably 1.

The above compounds of the invention wherein R_5 is $-NH-$ can be prepared from N-(9-fluorenylmethoxy-carbonyloxy)-succinimide (Fmoc-OSu) or N-(2-sulfo-9-fluorenylmethoxy-carbonyloxy)-succinimide (FMS-OSu) substituted by $-NH_2$ in the fluorene ring (depicted in Scheme 7, page a, first row, first column), by reacting the amino group with the activated PEG-OH (e.g. PEG-O-CO-Cl) or an activated derivatized PEG such as PEG-carboxylate (PEG-COOH, e.g. via PEG-CO-Cl), PEG-aldehyde (PEG-CHO), PEG-isocyanate (PEG-N=C=O), PEG-isothiocyanate (PEG-N=C=S), 2,4-dichloro-6-S-PEG-1,3,5-triazine, 2,4-dichloro-6-NH-PEG-1,3,5-triazine, or 2,4-dichloro-6-O-PEG-1,3,5-triazine (Scheme 7, page a, right column) in order to obtain the derivatives wherein $-R_6$ -PEG is as presented in Scheme 7 (page a, middle column).

In one preferred embodiment of the invention, the conjugate of the invention is a $(PEG-Fmoc)_n$ -peptide/protein, and the starting compound for their preparation is the maleimido derivative of Fmoc-OSu, herein designated Precursor 7 or MAL-Fmoc-NHS or MAL-Fmoc-OSu, of the formula depicted in Scheme 3.

In a most preferred embodiment of the invention, the conjugate of the invention is a $(PEG-FMS)_n$ -peptide/protein, and the starting compound for their preparation is the maleimido derivative of FMS-OSu, herein designated Precursor 8 or MAL-FMS-NHS or MAL-FMS-OSu, of the formula depicted in Scheme 3.

Two possible pathways for the pegylation of target peptides/proteins and preparation of the $(PEG-Fmoc)_n$ -peptide/protein or $(PEG-FMS)_n$ -peptide/protein conjugates are provided by the invention, as depicted in Scheme 6. Both pathways are two-step procedures.

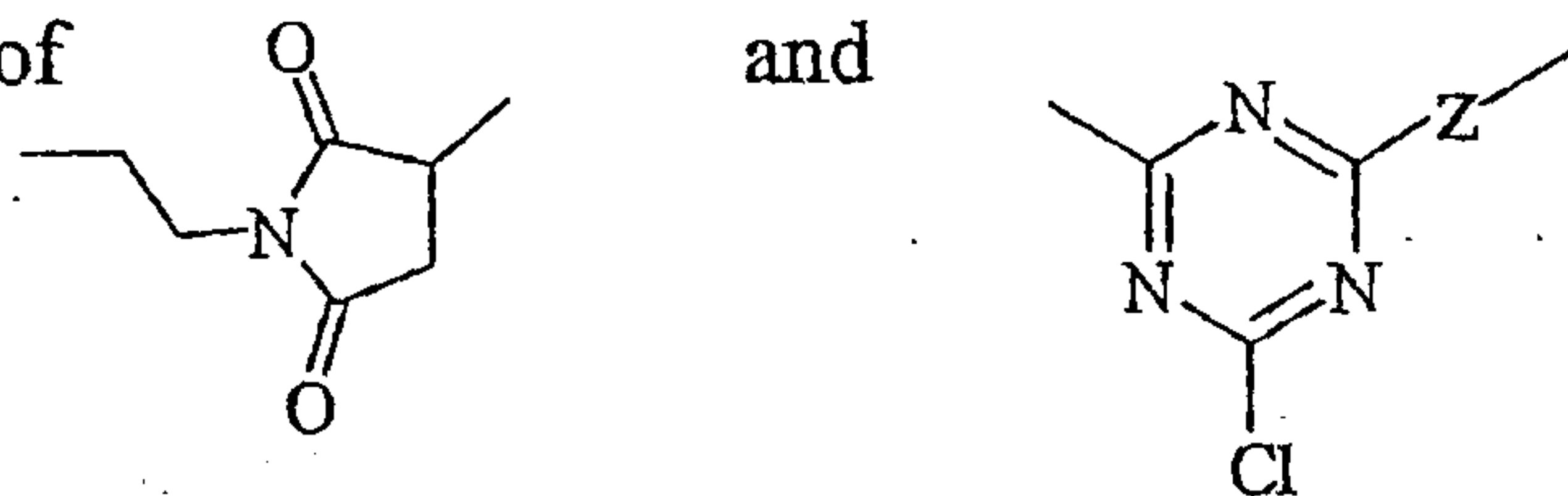
According to one pathway, MAL-FMS-NHS or MAL-Fmoc-NHS is first attached to the amine component of the target peptide/protein, thus obtaining a MAL-FMS-peptide/protein or MAL-Fmoc-peptide/protein conjugate, and then substituting PEG-SH for the maleimide moiety, producing the $(PEG-FMS)_n$ -peptide/protein or $(PEG-Fmoc)_n$ -peptide/protein conjugate, respectively.

In the second pathway, MAL-FMS-NHS or MAL-Fmoc-NHS is first reacted with PEG-SH, thus forming a PEG-FMS-NHS or PEG-Fmoc-NHS conjugate, and

then reacting it with the amine component of the target peptide or protein resulting in the desired (PEG-FMS)_n-peptide/protein or (PEG-Fmoc)_n-peptide/protein conjugate, respectively. This pathway is suitable for sulfhydryl- or disulfide-containing peptides and proteins.

5 The compounds wherein R₅ is -NH- and R₆ is -CO-NH- or -CS-NH- can be prepared from Fmoc-OSu or FMS-OSu substituted by -N=C=O or -N=C=S in the fluorene ring (depicted in Scheme 7, page C, first row and last rows, first column), respectively, by reaction with PEG-NH₂. No activation is necessary because these Fmoc/FMS species are already activated.

10 In a further embodiment of the invention, R₅ is -S-; and R₆ is selected from the group consisting of

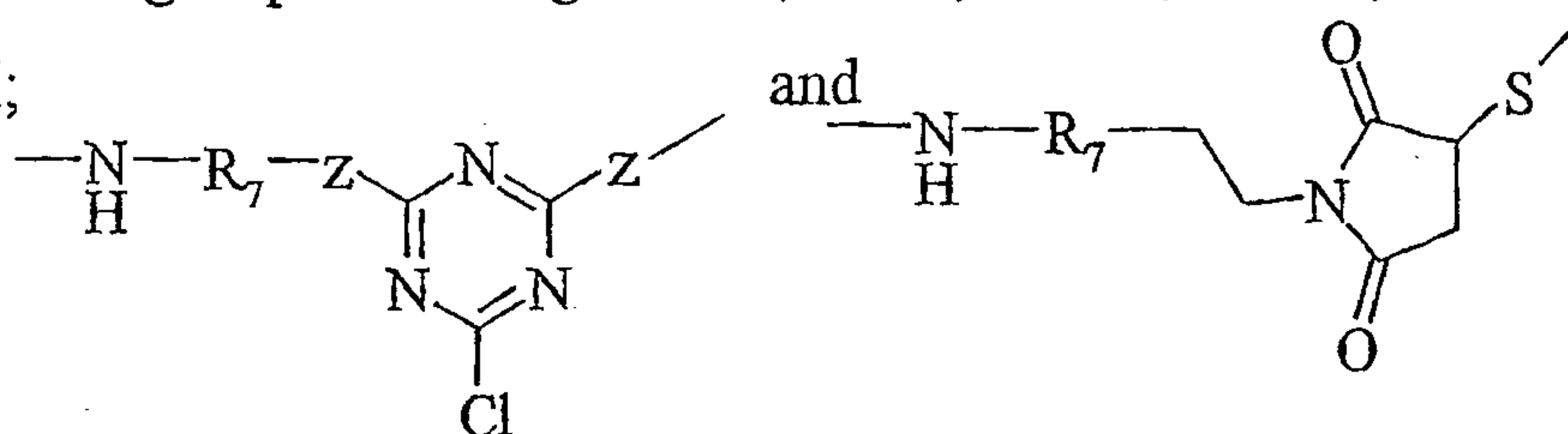


15 wherein Z is O, S or NH.

The above compounds of the invention wherein R₅ is -S- can be prepared from Fmoc-OSu or FMS-OSu substituted by -SH in the fluorene ring (depicted in Scheme 7, page b, second row, first column), by reaction with PEG-maleimide of the formula shown in Scheme 7, page b, second row, right column), thus obtaining a
 20 pegylated compound of the invention wherein the PEG moiety is linked to the fluorene ring through a residue as depicted in Scheme 7, page b, second row, middle column, or by reaction with 2,4-dichloro-6-S-PEG-1,3,5-triazine, 2,4-dichloro-6-NH-PEG-1,3,5-triazine, or 2,4-dichloro-6-O-PEG-1,3,5-triazine.

In yet another embodiment, in the pegylated drug of the invention, R_5 is $-CO-$; R_6 is selected from the group consisting of $-O-$; $-NH-$; $-NH-R_7-COO-$; $-NH-R_7-$; $-NH-R_7-CO-NH-$;

5



Z is O , S or NH ; and

R_7 is selected from the group consisting of C1-C18 straight or branched alkylene, phenylene, an oxyalkylene radical having 3-18 carbon atoms in the backbone, a residue of a peptide containing 2-10 amino acid residues, and a residue of a saccharide containing 1-10 monosaccharide residues.

The above compounds of the invention wherein R_5 is $-CO-$ can be prepared from Fmoc-OSu or FMS-OSu substituted by $-COOH$ in the fluorene ring (depicted in Scheme 7, page b, third row, first column). When R_6 is $-O-$ or $-NH-$, the reaction will occur with PEG (PEG-OH) or PEG-amine (PEG-NH₂), respectively, thus obtaining a pegylated compound wherein the PEG moiety is linked to the fluorene ring through a residue $-CO-O-$ or $-CO-NH-$, respectively, as depicted in Scheme 7, page b, middle column (3rd and 4th rows).

The compounds of the invention wherein R_5 is $-CO-$ and R_6 is $NH-R_7-COO-$ can be prepared from Fmoc-OSu or FMS-OSu substituted by $-COOH$ in the fluorene ring, by reaction with $H_2N-R_7-CO-OtBu$ using a coupling reagent (e.g. DCC/HOBt, or PyBOP(benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate)/triethylamine), removal of the *t*Bu protecting group under acidic conditions (e.g. trifluoroacetic acid or HCl in dioxane), activation of the free carboxyl group by triphosgen and reaction of the $-NH-R_7-COCl$ formed with PEG-OH to obtain the $-CO-NH-R_7-CO-O-PEG$ derivative.

The compounds of the invention wherein R_5 is $-CO-$ and R_6 is $NH-R_7-NH$ can be prepared from Fmoc-OSu or FMS-OSu substituted by $-COOH$ in the fluorene ring, by reaction with $H_2N-R_7-NH-tBu$ using a coupling reagent and

removing the *t*Bu protecting group as described above, reacting the free amino group with PEG-OSu to obtain the -CO-NH-R₇-NH-PEG derivative.

The compounds of the invention wherein R₅ is -CO- and R₆ is NH-R₇-CO-NH- can be prepared from Fmoc-OSu or FMS-OSu substituted by -COOH in the fluorene ring, by reaction with H₂N-R₇-CO-NH-*t*Bu using a coupling reagent and removing the *t*Bu protecting group as described above, activating the free carboxyl group with DCC/NHS and reacting of the -NH-R₇-CO-N-hydroxysuccinimide ester formed with PEG-NH₂ to obtain the -CO-NH-R₇-CO-NH-PEG derivative.

The compounds of the invention wherein R₅ is -CO- and R₆ is -NH-R₇-NH-Z-(4-chloro-6-Z-PEG-1,3,5-triazin-2-yl) can be prepared from Fmoc-OSu or FMS-OSu substituted by -COOH in the fluorene ring, by reaction with H₂N-R₇-NH-*t*Boc or H₂N-R₇-O-*t*Bu or H₂N-R₇-S-trityl using a coupling reagent and removing the *t*Boc, *t*Bu or trityl protecting group as described above, reacting the free NH₂, OH or SH group with 2,4,6-trichloro-1,3,5-triazine, and further reacting the -NH-R₇-NH[O or S]-(4-chloro-6-NH[O or S]-1,3,5-triazin-2-yl) thus formed with PEG-NH₂, PEG-OH or PEG-SH to obtain the corresponding -CO--NH-R₇-NH[O or S]-(4-chloro-6-NH[O or S]-PEG-1,3,5-triazine derivative.

The compounds of the invention wherein R₅ is -CO- and R₆ is NH-R₇-ethylene-succinimido can be prepared from Fmoc-OSu or FMS-OSu substituted by -COOH in the fluorene ring, by reaction with H₂N-R₇-ethylene-maleimide using a coupling reagent (e.g. DCC/HOBt, PyBoP/Triethylamine) followed by reaction of the maleimide moiety with PEG-SH at pH 6-8 to obtain the -CO-NH-R₇-ethylene-succinimido-S-PEG derivative.

The pegylated-Fmoc/FMS-drugs of the invention are then prepared from these intermediates by the one-step procedure described in Example 5 hereinafter.

In yet a further embodiment of the invention, R₅ is -CH₂-; and R₆ is -(CH₂)_n-S- or -(CH₂)_n-NH-, wherein n is 0 to 18, preferably 1.

The above compounds of the invention wherein R₅ is -CH₂- and R₆ is -CH₂-S- or -R₆ is -CH₂-NH- can be prepared from Fmoc-OSu or FMS-OSu substituted by -COH in the fluorene ring (depicted in Scheme 7, page c, 4th row, first column),

by reaction with PEG-SH or PEG-NH₂ followed by reduction with NaBH₃, respectively.

The above compounds of the invention wherein R₅ is -CH₂- and R₆ is -(CH₂)_n-S- or R₆ is -(CH₂)_n-NH- can be prepared from Fmoc-OSu or FMS-OSu substituted by -(CH₂)_n-Hal in the fluorene ring, wherein Hal is F, Cl, Br or I (depicted in Scheme 7, page d, 3rd row, first column), by reaction with PEG-NH₂ or PEG-SH, thus obtaining a pegylated compound of the invention wherein the PEG moiety is linked to the fluorene ring through a residue as depicted in Scheme 7, page d, middle column, 3rd and 4th rows, respectively.

10 The pegylated-Fmoc/FMS-drugs of the invention are then prepared from the above intermediates by the one-step procedure described in Example 5 hereinafter.

In still another embodiment, R₅ is -SO₂- and R₆ is -O-, -NH- or -CH₂-CH₂-S. The compounds wherein R₆ is -O- or -NH- can be prepared from Fmoc-OSu or FMS-OSu substituted by -SO₂Cl in the fluorene ring (depicted in Scheme 7, page c, first column, 3rd row), by reaction with PEG-OH or PEG-NH₂, respectively. The pegylated-Fmoc/FMS-drugs of the invention are then prepared from these intermediates by the one-step procedure described in Example 5 hereinafter.

20 The compounds wherein R₆ is -CH₂-CH₂-S, can be prepared from Fmoc-OSu or FMS-OSu substituted by -SO₂CH=CH₂ in the fluorene ring (depicted in Scheme 7, page c, first column, 2nd row), by reaction with PEG-SH. The pegylated-Fmoc/FMS-drugs of the invention are then prepared from these intermediates by the two-step procedure described in Examples 16 or 17 hereinafter.

25 In yet still another embodiment, R₅ is -PO₂- and R₆ is -O- or -NH-. These compounds can be prepared from Fmoc-OSu or FMS-OSu substituted by -PO₂Cl in the fluorene ring (depicted in Scheme 7, page d, first column, first row), by reaction with PEG-OH or PEG-NH₂, respectively. The pegylated-Fmoc/FMS-drugs of the invention are then prepared from these intermediates by the one-step procedure described in Example 5 hereinafter.

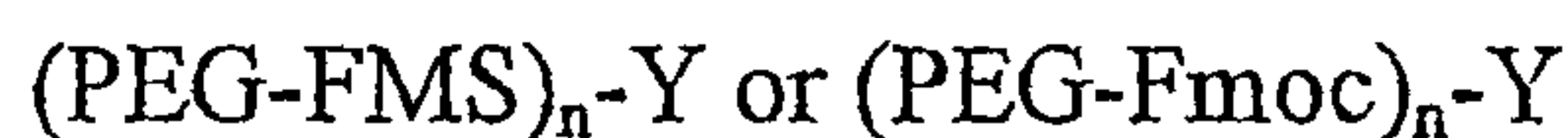
30 According to the present invention, Y is a moiety of a drug bearing at least one functional group selected from free amino, carboxyl, hydroxyl, phosphate

and/or mercapto. In a more preferred embodiment of the invention, the drug contains at least one free amino group and is a peptide or a protein drug or a non-peptidic drug.

In one embodiment of the invention, the drug is a non-peptidic drug that contains at least one amino group and the invention relates to PEG-Fmoc and PEG-FMS conjugates thereof. Non-peptidic drugs that are amenable to the technology of the invention include antibiotic aminoglycosides such as gentamicin and amphotericin B, and antineoplastic drugs such as aminolevulinic acid, daunorubicin and doxorubicin.

In a more preferred embodiment of the invention, the drug containing at least one amino group is a peptide or protein drug, most preferably a peptide or protein of low or medium molecular weight, that can be used as a drug for human or veterinary use.

Thus, further provided by the invention are pegylated drug conjugates PEG-FMS-Y and PEG-Fmoc-Y herein identified by the formulas:



wherein Y is a moiety of a peptide or protein drug, n is an integer of at least one, preferably 1 or 2, and Y is linked to the FMS or Fmoc radical through at least one amino group. In the most preferred embodiment the conjugate is $(\text{PEG-FMS})_n\text{-Y}$ and the PEG moiety is PEG₄₀₀₀₀.

Examples of peptides and proteins Y that can be pegylated according to the invention include, but are not limited to, insulin, an interferon such as IFN- α 2, a PYY agonist such as the peptide PYY₃₋₃₆, an exendin such as exendin-3 and exendin-4 and exendin analogues and agonists, atrial natriuretic peptide (ANP), human growth hormone (hGH), erythropoietin, TNF- α , calcitonin, gonadotropin releasing hormone (GnRH) or an analogue thereof such as leuprolide and D-Lys⁶-GnRH, hirudin, glucagon, and a monoclonal antibody fragment such as anti-TNF- α monoclonal antibody fragment.

In one preferred embodiment of the invention, the peptidic drug is insulin and the invention provides PEG-Fmoc-insulin and PEG-FMS-insulin conjugates

and pharmaceutical compositions comprising them for treatment of diabetes mellitus and hyperglycemia. Examples of such conjugates are the (PEG₅₀₀₀-Fmoc)₁-insulin, (PEG₅₀₀₀-Fmoc)₂-insulin and PEG₄₀₀₀₀-FMS-insulin conjugates.

5 In another preferred embodiment of the invention, the drug is an exendin or an exendin agonist.

In a more preferred embodiment, the drug is exendin-4 of the sequence represented by SEQ. ID. NO:1:

HGEGTFTSDL SKQMEEEEAVR LFIEWLKNNGG PSSGAPPPS-NH₂

10 In another preferred embodiment, the drug is exendin-3 of the sequence represented by SEQ. ID. NO:2:

HSDGTFITSDL SKQMEEEEAVR LFIEWLKNNGG PSSGAPPPS-NH₂

In a further preferred embodiment, the drug is an exendin agonist defined herein as a compound that mimics the activities of exendin-3 or exendin-4 by binding to the receptor(s) at which exendin-3 or exendin-4 exerts its actions which are beneficial as insulinotropic and in the treatment of diabetes mellitus or by mimicking the effects of exendin on increasing urine flow, increasing urinary sodium excretion and/or decreasing urinary potassium concentration, by binding to the receptor(s) where exendins cause these effects. Preferably, the exendin agonist is selected from the group of insulinotropic exendin-4 fragments and analogues consisting of exendin agonists represented by SEQ ID NO:3 to SEQ ID NO: 10:

15

20

exendin-4 (1-31) HGEGTFTSDLSKQMEEEEAVRLFIEWLKNNGGP

[SEQ ID No:3];

Y³¹exendin-4(1-31) HGEGTFTSDLSKQMEEEEAVRLFIEWLKNNGGY

[SEQ ID No:4];

25 exendin-4 (1-30) HGEGTFTSDLSKQMEEEEAVRLFIEWLKNNGG

[SEQ ID No:5];

exendin-4 (1-30) amide HGEGTFTSDLSKQMEEEEAVRLFIEWLKNNGG-NH₂ [SEQ ID No:6];

30 exendin-4 (1-28) amide HGEGTFTSDLSKQMEEEEAVRLFIEWLKN-NH₂ [SEQ ID No:7]; L¹⁴,

F²⁵ exendin-4 amide HGEGTFTSDLSKQLEEEAVRLFIEFLKNGG
PSSGAPPPS-NH₂ [SEQ ID No:8];

L¹⁴,F²⁵ exendin-4 (1-28) amide HGEGTFTSDLSKQLEEEAVRLFIEFLKN-
NH₂ [SEQ ID No:9]; and

5 L¹⁴,A²²,F²⁵ exendin-4 (1-28) amide HGEGTFTSDLSKQLEEEAVRLAIE
FLKN-NH₂ [SEQ ID No:10].

According to this embodiment, the invention provides PEG-Fmoc-
exendin/exendin agonist and PEG-FMS-exendin/exendin agonist conjugates and
pharmaceutical compositions comprising them for prevention of hyperglycemia and
10 for treatment of diabetes mellitus selected from the group consisting of non-insulin
dependent diabetes mellitus, insulin-dependent diabetes mellitus, and gestational
diabetes mellitus. In a most preferred embodiment, the pegylated exendin conjugate
of the invention is PEG₄₀-FMS-exendin-4.

In a further preferred embodiment of the invention, the peptidic drug is an
15 interferon, preferably IFN- α , and the invention provides PEG-Fmoc-IFN- α and
PEG-FMS-IFN- α conjugates and pharmaceutical compositions comprising them for
treatment of diseases treatable by IFN- α , particularly viral diseases, more
particularly hepatitis B or C, both as sole therapy or in conjunction with an antiviral
agent such as ribavirin, or for treatment of cancer, e.g. transitional cell carcinoma,
20 the most common type of bladder cancer, ovarian cancer, pancreatic cancer
melanoma, non-Hodgkin's lymphoma, hairy cell leukemia, and AIDS-related
Kaposi's sarcoma, both as sole therapy or in conjunction with a cytotoxic agent such
as carboplatin and/or cyclophosphamide. In a more preferred embodiment, the
conjugate is (PEG₄₀-FMS)₂-IFN α 2 or, most preferably, PEG₄₀-FMS-IFN α 2.

25 In still another preferred embodiment of the invention, the peptidic drug is a
PYY agonist, herein defined as a molecule that has a PYY- or PYY[3-36]-like
biological activity such as reducing food intake in mammals, and acts by a
mechanism similar to that of PYY and PYY[3-36], for example by binding to the
Y2 receptor. The PYY agonist is preferably an agonist specific for the Y2 receptor

and is preferably a peptide containing, at a minimum, the sequence of amino acids 25-36 of PYY, most preferably the sequence 3-36 of PYY.

In one embodiment of the invention, the PYY agonist is the 36-mer peptide PYY of the sequence represented by SEQ ID NO: 11:

5 YPIKPEAPGEDASPEELNRYASYLRHYLNLVTRQRY-NH₂

In a more preferred embodiment of the invention, the PYY agonist is the peptide PYY[3-36] of the sequence represented by SEQ ID NO: 12:

IKPEAPGEDASPEELNRYASYLRHYLNLVTRQRY-NH₂

According to this embodiment, the invention provides PEG-Fmoc-PYY
10 agonist and PEG-FMS-PYY agonist conjugates and pharmaceutical compositions comprising them for particularly for reduction of food intake, for inducing weight loss and for the treatment of diseases or disorders which can be alleviated by reduction of food intake such as obesity, hypertension, dyslipidemia, cardiovascular risk, insulin-resistance, and diabetes mellitus (particularly type II diabetes). In a
15 preferred embodiment, the pegylated PYY agonist of the invention is PEG₄₀-FMS-PYY₃₋₃₆.

In another preferred embodiment, the peptidic drug is human growth hormone (hGH) and the invention provides PEG-Fmoc-hGH and PEG-FMS-hGH conjugates and pharmaceutical compositions comprising them for treatment of
20 conditions and disorders treatable by hGH, particularly for treatment of children of pathologically short stature and as anti-aging agent. In preferred embodiments, the pegylated hGH conjugates of the invention are (PEG₄₀-FMS)₂-hGH and PEG-FMS-hGH.

In a further preferred embodiment of the invention, the peptidic drug is atrial
25 natriuretic peptide (ANP) or an analog thereof, particularly the cyclic 28-amino acid ANP of the sequence represented by SEQ ID NO:13, as follows:

Ser-Leu-Arg-Arg-Ser-Ser-[Cys7-Phe-Gly-Gly-Arg-Met-Asp-Arg-Ile-Gly-Ala-Gln-Ser-Gly-Leu-Gly-Cys23]-Asn-Ser-Phe-Arg-Tyr

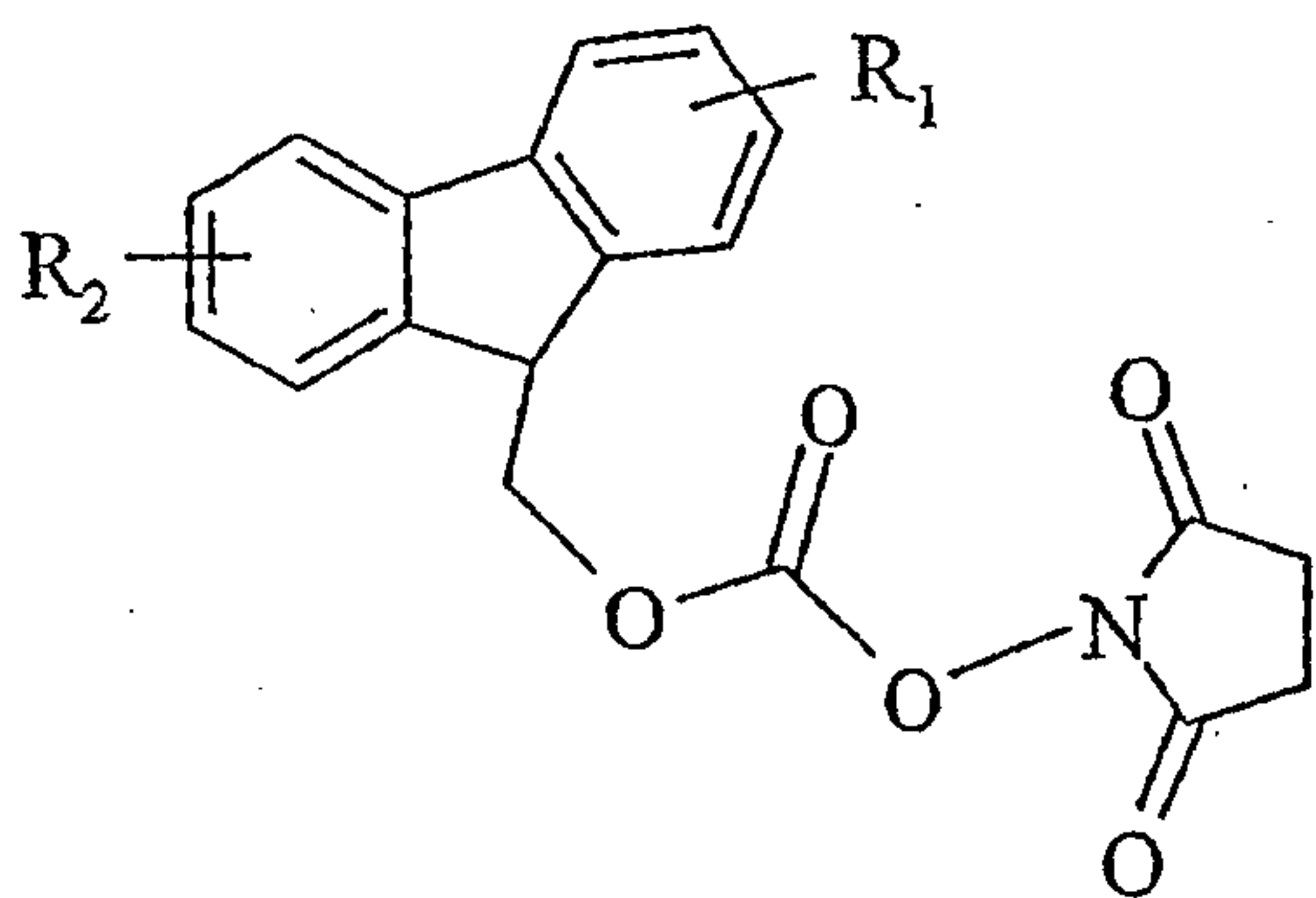
According to this embodiment, the invention provides PEG-Fmoc-ANP and
30 PEG-FMS-ANP conjugates and pharmaceutical compositions comprising them for

treatment of conditions and disorders treatable by the invention provides PEG-Fmoc-ANP and PEG-FMS-ANP conjugates and pharmaceutical compositions comprising them for treatment of conditions and disorders treatable by natriuretic peptides and variants thereof, particularly treatment of cardiovascular diseases, congestive heart failure, hypertension, acute kidney failure and adult respiratory distress syndrome (ARDS). In one preferred embodiment of the invention, the pegylated ANP is the PEG₄₀-FMS-ANP conjugate.

Also included in the scope of the invention are pharmaceutically acceptable salts of the pegylated conjugates of the invention. As used herein, the term "salts" refers to both salts of carboxyl groups and to acid addition salts of amino groups of the drug, e.g. peptide or protein, molecule. Salts of a carboxyl group may be formed by means known in the art and include inorganic salts, for example, sodium, calcium, ammonium, ferric or zinc salts, and the like, and salts with organic bases such as those formed for example, with amines, such as triethanolamine, arginine, or lysine, piperidine, procaine, and the like. Acid addition salts include, for example, salts with mineral acids such as, for example, hydrochloric acid or sulfuric acid, and salts with organic acids, such as, for example, acetic acid or oxalic acid.

The present invention further relates to methods for the preparation of the pegylated conjugates of the invention and to several novel precursor compounds used in these methods.

Thus, the present invention also relates to a precursor of the formula:



wherein:

R₁ is a radical of the formula -R₅-R₆-B;

R_2 is H or $-\text{SO}_3\text{H}$ at position 2 of the fluorene ring;

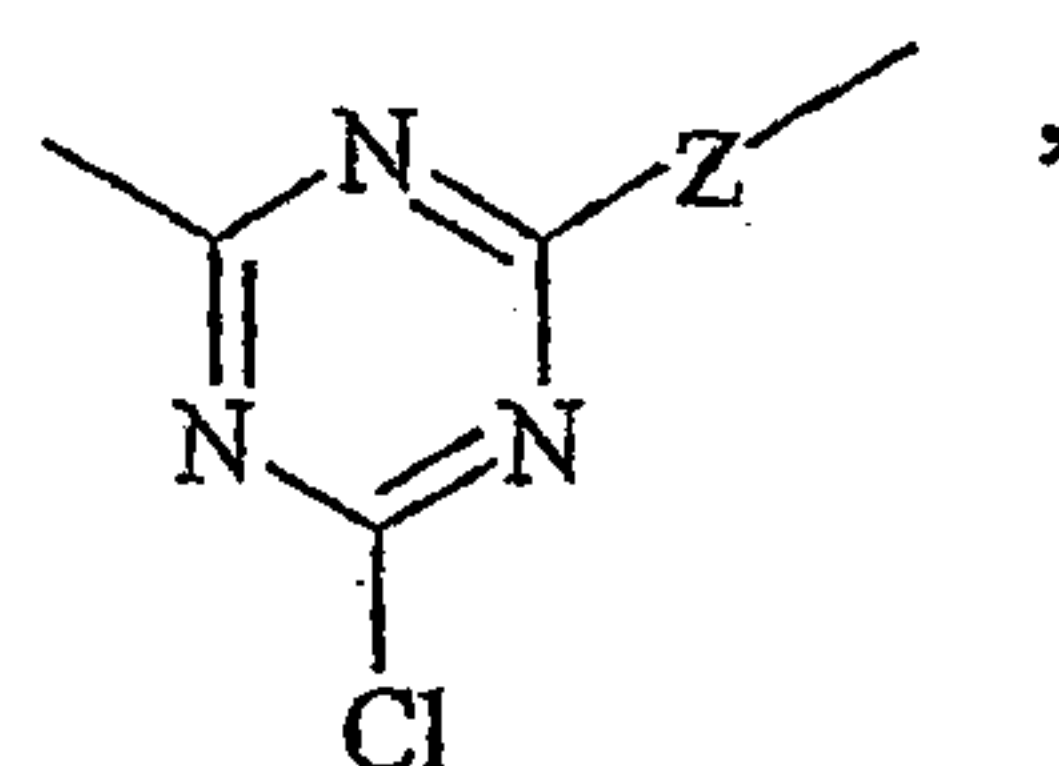
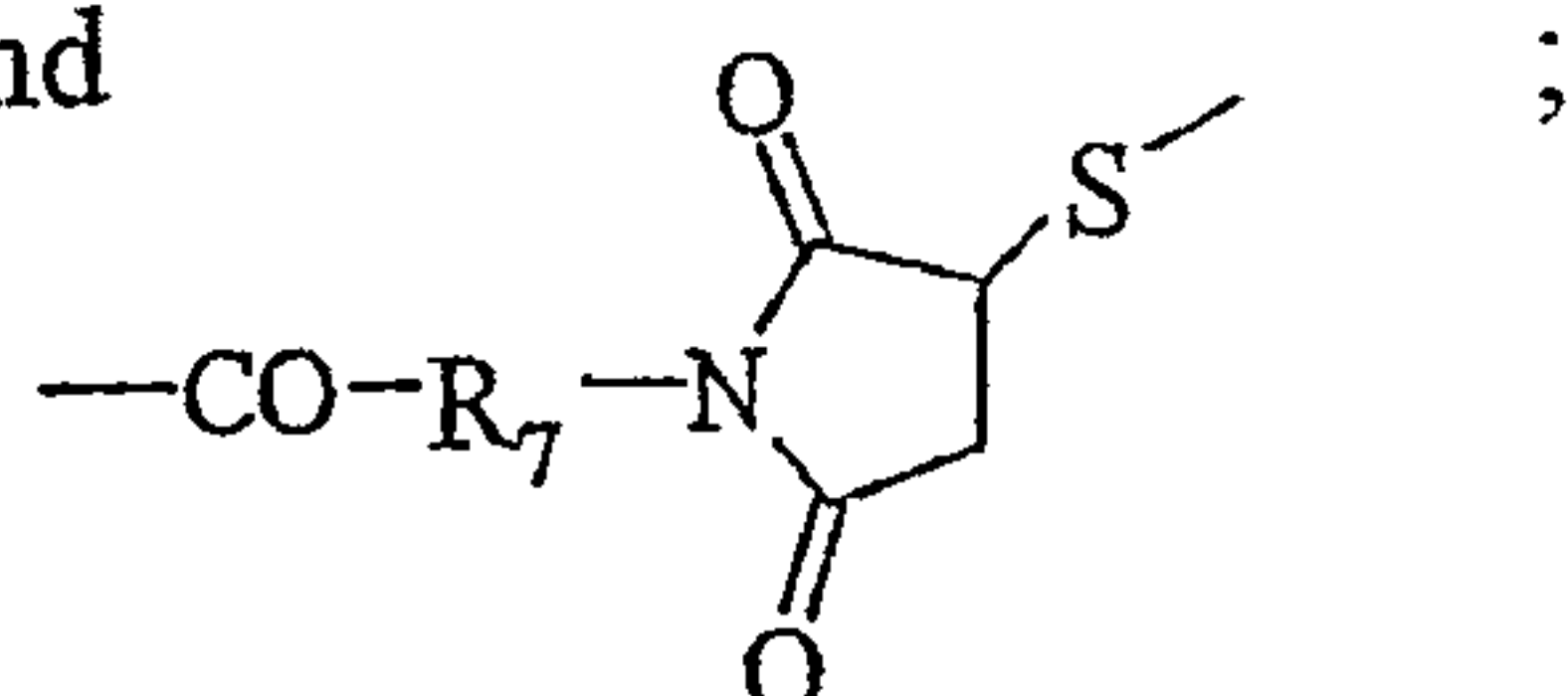
B is maleimido, $-\text{S}-\text{CO}-\text{CH}_3$ or a PEG moiety;

R_5 is selected from the group consisting of $-\text{NH}-$, $-\text{S}-$, $-\text{CO}-$, $-\text{COO}-$, $-\text{CH}_2-$, $-\text{SO}_2-$, $-\text{SO}_3-$, $-\text{PO}_2-$, and $-\text{PO}_3-$; and

5 R_6 is a bond or a radical by which the maleimido, $-\text{S}-\text{CO}-\text{CH}_3$ or PEG moiety is attached to R_5 .

In one preferred embodiment, R_5 is $\text{NH}-$; R_6 is selected from the group consisting of $-\text{CO}-$, $-\text{COO}-$, $-\text{CH}_2-$, $-\text{CH}(\text{CH}_3)-$, $\text{CO}-\text{NH}-$, $-\text{CS}-\text{NH}-$, $-\text{CO}-\text{CH}_2-\text{NH}-$, $-\text{CO}-\text{CH}(\text{CH}_3)-\text{NH}-\text{CO}-$, $-\text{CO}-\text{CH}_2-\text{NH}-\text{CO}-\text{NH}-$, $-\text{CO}-\text{R}_8-$;

10 and



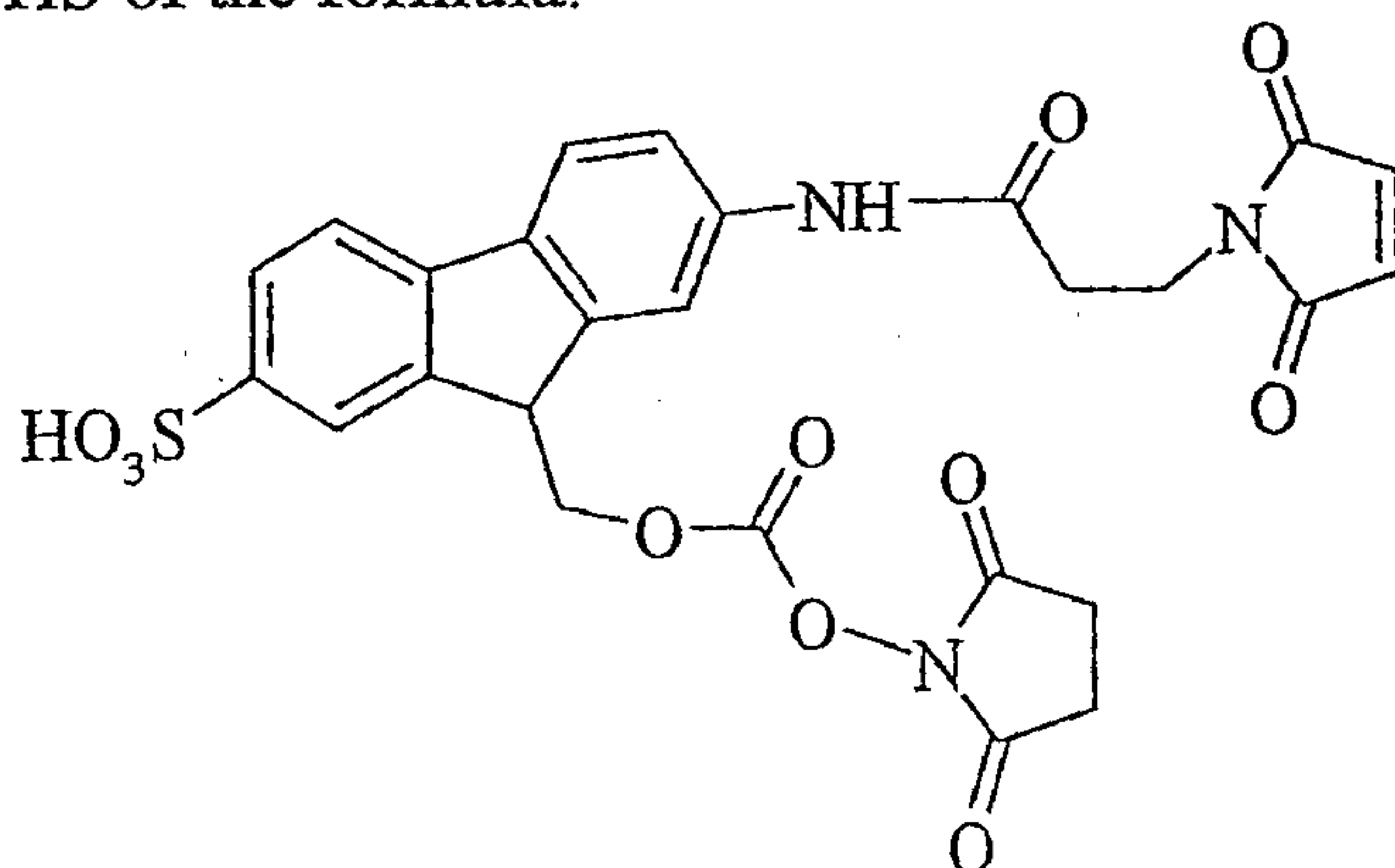
Z is O, S or NH;

15 R_7 is selected from the group consisting of C1-C18 straight or branched alkylene, phenylene, an oxyalkylene radical having 3-18 carbon atoms in the backbone, a residue of a peptide containing 2-10 amino acid residues, and a residue of a saccharide containing 1-10 monosaccharide residues; and

20 R_8 is a C1-C8 straight or branched alkylene, preferably ethylene, when B is maleimido or $-\text{S}-\text{CO}-\text{CH}_3$.

In one preferred embodiment, the invention relates to the novel *Precursors 1-7*, whose formulas are depicted in Schemes 1 and 3. In the most preferred embodiment, the invention relates to the compound herein identified as *Precursor 8* or MAL-FMS-NHS of the formula:

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Precursor 8 is the compound N-[2-(maleimido-propionyl amino)-7-sulfo-fluoren-9-yl-methoxycarbonyloxy]-succinimide [or 9-hydroxymethyl-2-(amino-3-maleimidopropionate)-7-sulfo-fluorene-N-hydroxysuccinimide] and is also herein identified as MAL-FMS-OSu.

5 MAL-FMS-NHS is a water-soluble hetero-bifunctional reagent, consisting of a sulfonated fluorenyloxycarbonyl N-hydroxysuccinimide ester that reacts with peptide and protein amino groups. A maleimide group was attached to the fluorenyl backbone to enable coupling to sulfhydryl-containing PEG, most preferably PEG₄₀₀₀₀-SH.

10 Thus, further provided by the invention are precursor drug conjugates herein identified by the formula:



wherein Y is a moiety of a drug, more preferably a peptide or protein drug, n is an integer of at least one, preferably 1 or 2, and Y is linked to the FMS or Fmoc radical through an amino group.

The present invention also provides a method for the preparation of a conjugate (PEG-Fmoc)_n-Y, wherein Y is a moiety of a drug, more preferably a peptide or protein drug, n is an integer of at least one, preferably 1 or 2, and Y is linked to the Fmoc radical through an amino group, which comprises:

- 20 (i) reacting a drug Y, e.g. a peptide or protein drug, with at least one equivalent of Precursor 7, thus obtaining a conjugate (MAL-Fmoc)_n-Y; and
 (ii) reacting the conjugate (MAL-Fmoc)_n-Y with PEG-SH.

In a most preferred embodiment, the invention relates to a method for preparation of a conjugate (PEG-FMS)_n-Y, wherein Y is a moiety of a drug, more preferably a peptide or protein drug, n is an integer of at least one, preferably 1 or 2, and Y is linked to the FMS radical through an amino group, which comprises:

- 25 (i) reacting a drug Y, e.g. a peptide or protein drug, with at least one equivalent of MAL-FMS-NHS, thus obtaining a conjugate (MAL-FMS)_n-Y; and
 (ii) reacting the conjugate (MAL-FMS)_n-Y with PEG-SH, thus obtaining
 30 the conjugate (PEG-FMS)_n-Y.

In another most preferred embodiment, the invention relates to a method for preparation of a conjugate $(\text{PEG-FMS})_n\text{-Y}$, which comprises:

(i) reacting MAL-FMS-NHS with PEG-SH, thus obtaining a conjugate PEG-FMS-NHS; and

5 (ii) reacting the drug Y, e.g. a peptide or protein drug, with at least one equivalent of the conjugate PEG-FMS-NHS, thus obtaining the conjugate $(\text{PEG-FMS})_n\text{-Y}$.

The PEG-SH reagent is preferably $\text{PEG}_{40}\text{-SH}$, wherein the PEG moiety is a branched PEG moiety of molecular weight 40,000 Da.

10 As mentioned in the Background section herein, the pegylation technique has been extensively used for modifying molecules, in particular peptide and protein drugs, in an attempt to improve some of its characteristics such as improved stability and solubility, reduced immunogenicity, reduced proteolysis, reduced toxicity, reduced clearance by the kidneys, improved bioavailability, and extended
15 circulating life thus less frequent dosing being required. However, one of the main problems of pegylation is that covalent bonding between the PEG moiety and the drug most often causes loss of biological activity or drastic decrease of pharmacological potency of the drug. For this reason, pegylation is used more for high-molecular weight proteins, that are less likely to be inactivated by the reaction
20 with PEG, but is less frequent for peptides and low-molecular weight proteins.

Theoretically, decreased bioactivity of peptides and proteins by pegylation can be overcome by linking the PEG-chains via a chemical bond sensitive to mild alkaline and/or acid hydrolysis, or enzymatically cleavable by serum proteases or esterases. Obviously, an inconsistent rate of hydrolysis would render such an
25 approach impractical. A prerequisite condition is therefore that the hydrolysis of the PEG-chains from the conjugate take place at a slow rate and in a homogenous fashion under the strictly homeostatic pH and temperature conditions of the mammalian circulatory system.

Previously, we have prepared 2-sulfo-9-fluorenylmethoxycarbonyl-N-
30 hydroxysuccinimide (FMS-OSu) (Gershonov et al., 1999, 2000; Shechter et al.,

2001, 2001a) as a reversible protein modifier. Protein-linked FMS moieties undergo slow and spontaneous hydrolysis under physiological conditions generating the unmodified parent molecule. Hydrolysis of FMS-protein conjugates at 37°C in normal human serum, in aqueous buffers of pH 8.5, or in the circulatory system *in vivo*, takes place with a $t_{1/2}$ of 5-7 hrs. Using the FMS moiety as the scaffold for the suggested reversible pegylation technology enables the hydrolysis rates of various PEG-FMS-protein conjugates to be predicted. The $t_{1/2}$ for the hydrolysis of modified small molecules and PEG-conjugated polypeptides and proteins falls within a relatively narrow range of 8-14h. The constant hydrolysis rate for PEG-FMS-protein conjugates is due to the β -elimination reaction, which occurs at position 9 of the fluorenyl moiety, being solely dependent on the pH of the surrounding medium. Thus, in contrast to what occurs in approaches based on enzymatic hydrolytic bond cleavage, similar PEG-FMS hydrolysis rates are expected from all conjugates, regardless of the identity of the protein/peptide moiety conjugated. Using the present approach one can further control the hydrolysis rate by substitution of the fluorenyl moiety with electron-withdrawing or electron-inducing groups that increase or decrease, respectively, the hydrolysis rate. The number of PEG-FMS chains attached to the drug should also affect the rate at which the native drug is released.

20 The sulfonated fluorene moiety is not toxic, as previously shown (Shechter et al., 2001). High molecular-weight PEG is known to be safe in terms of toxicity and immunogenicity and is widely used in the food, cosmetic and pharmaceutical industries (Working et al., 1997; Roberts et al., 2002).

25 Thus, the present invention provides a procedure herein designated "reversible pegylation". In this new conceptual approach, that was implemented according to the invention with low molecular-weight polypeptides and proteins, the PEG moiety is not attached directly to the drug, as in the standard pegylation procedure, but rather the PEG moiety is attached directly or through a linker to a moiety of formula (i) to (iv) herein, and the drug is attached to another position of the moiety (i) to (iv). Said moiety (i) to (iv), preferably the Fmoc or FMS moiety

30

(i), is highly sensitive to bases and is removable under mild basic conditions. Thus, in this way, a prodrug is obtained that is inactive, but undergoes transformation into the active drug under the physiological conditions of the body. The prodrug has an extended circulation life but the PEG moiety is removed together with the Fmoc or
5 FMS moiety and the drug recovers its full pharmacological potency.

This novel approach enables the desirable pharmacological features associated with pegylation to be conferred on low molecular-weight peptide and protein drugs that would otherwise have been fully or partially inactivated by this technique. A pharmacologically 'silent' conjugate that is 'trapped' in the circulatory
10 system releases the covalently-linked parent peptide or protein, with a desirable pharmacokinetic profile. This new approach is expected to extend the life-time, bioavailability and efficacy of existing peptide drugs, and to extend the same in known peptide drugs and peptide drug candidates that may yet be discovered.

This new technology has been successfully tested according to the invention
15 on several peptide and protein drugs. In addition to prolonging life-times *in vivo*, the inactive but reactivatable PEG-protein conjugate has the profound advantage of maintaining a low circulating level of the active protein drug at any time point after administration. In this way, a well-known risk of the presence of a "burst" of toxifying or desensitizing drug in the circulation is avoided.

20 As mentioned above, in theory one can design PEG chains that can be released from PEG-protein conjugates by serum proteases or esterases. However, rapid or unpredictable rate of release is not useful. A prerequisite condition with this new technology is that the PEG-chains should be hydrolyzed spontaneously from the conjugates at a slow, continuous and predictable rate. Release of PEG-
25 chains should occur over a prolonged period, thus maintaining the projected conjugates in the circulatory system, prior to removal of PEG-chains by hydrolysis. The present invention fulfills these requirements. For example, upon incubation in normal human serum at 37°C, PEG-chains are hydrolyzed from proteins, with a half-time of 8.0 ± 2 hrs. Rates of release are dictated exclusively by the nature of the

Fmoc-moiety (Fmoc/FMS), by the pH and the reactivity of the blood serum, and mammals maintain strict homeostasis with regards to these last two parameters.

According to the present invention, a pilot experiment included synthesis of a pegylated insulin. Upon incubation of (PEG₅₀₀₀-Fmoc)₁-insulin derivative at pH 8.5, or at normal human serum at 37°C, the lipogenic activity was restored with a half-life of 30±2 hrs. Regeneration of the lipogenic potency of bis-modified insulin, (PEG₅₀₀₀-Fmoc)₂-insulin, followed an additional lag period of 10±1 hrs.

A single subcutaneous administration of (PEG₅₀₀₀-Fmoc)₁-insulin in mice, lowered circulating glucose levels and the half time of return to normoglycemic values exceeded 6.7 fold that for native insulin. Following intraperitoneal administration of (PEG₅₀₀₀-Fmoc)₁-insulin, the return to normoglycemia was 3.4 fold slower than after administration of the native hormone. In sum, a prototype of a reversible PEG has been established. It undergoes slow spontaneous hydrolysis after conjugation, regenerating the non-modified parent drugs, at physiological conditions. Thus, the principal drawback of inactivating drugs by pegylation is solved by the technology of the present invention. The PEG-FMS moieties hydrolyze at faster rates as compared to PEG-Fmoc-moieties ($t_{1/2}$ =5-7 hrs, ref.). Compounds containing FMS-PEG moieties are more suitable when 2-5 PEG-chains are to be introduced into a peptide or a protein drug, for obtaining the desirable pharmacological features of the conjugates.

In another example of the present invention, it is shown herein that when native exendin-4 was subcutaneously administered at a dose of 4 µg /mouse, the blood glucose level (BGL) declined by 26-28% (from 140 mg/dl to 104-101 mg/dl), with the largest percent change in BGL occurring 0.5-1 h after administration. Glucose concentrations then returned to their initial levels with a $t_{1/2}$ of 3.7±0.3 h. Following the subcutaneous administration of PEG₄₀₀₀₀-FMS-exendin-4, the decrease in BGL took place at a more moderate rate. Circulating glucose reached its lowest concentration 8-12 hours after administration (92 mg/dl, 33%). Stable, low circulating glucose concentrations were then maintained for a further 12 hours.

Return to initial glucose levels took place with a $t_{1/2}$ of 30 ± 2 h, being 7.5 times longer than that obtained by the same dose of the native exendin-4.

In a further example of the present invention, it is shown herein that using a BIAcore binding assay, the *in vitro* rate of regeneration of native interferon was estimated to have a half-life of 65 hrs. Following subcutaneous administration to rats and monitoring circulating antiviral potency, active IFN α 2 levels peaked at 50 hrs, with substantial levels still being detected 200 hrs post administration. This value contrasts with a half-life of about 1 hr measured for unmodified interferon. The concentration of active IFN α 2 scaled linearly with the quantity injected. Comparing subcutaneous to intravenous administration of PEG₄₀-FMS-IFN α 2, we found that the long circulatory lifetime of IFN α 2 was affected both by the slow rate of absorption of the pegylated protein from the subcutaneous volume and by the slow rate of discharge from the PEG in circulation. A numerical simulation of the results was in good agreement with the results observed *in vivo*. The pharmacokinetic profile of this novel pegylated IFN α 2 conjugate combines a prolonged maintenance *in vivo* with the regeneration of active-native IFN α 2, ensuring ready access to peripheral tissues and thus an overall advantage over currently used formulations.

Peptide YY₃₋₃₆ (PYY₃₋₃₆) was recently shown to induce satiety in mice and humans. It is described herein that the satiety induced by subcutaneous administration of PYY₃₋₃₆ to fasting mice had a half-life of ~ 3 h. Pegylation of PYY₃₋₃₆ through a non-hydrolysable bond yielded an inactive conjugate but the conjugate of the invention PEG₄₀-FMS-PYY₃₋₃₆ gradually released unmodified PYY₃₋₃₆ under physiological conditions. Subcutaneous administration of PEG₄₀-FMS-PYY₃₋₃₆ to mice resulted in protracted satiety, with a half-life of ~ 24 h. PEG₄₀-FMS-PYY₃₋₃₆ can therefore serve as a long-acting prodrug of PYY₃₋₃₆, thereby providing a more practical means for controlling human obesity.

The PEG moiety according to the invention may be linear or branched PEG and has a molecular weight in the range of 200 to 200,000 Da, preferably up to 80,000 Da, more preferably 5,000-40,000 Da, and most preferably between about

20,000 Da and 40,000 Da. Preferably, the PEG moiety is a branched PEG molecule of 40,000 Da.

The prodrugs of the present invention are prepared using PEGylating agents, meaning any PEG derivative which is capable of reacting with a functional group such as, but not limited to, NH_2 , OH , SH , COOH , CHO , $-\text{N}=\text{C}=\text{O}$, $-\text{N}=\text{C}=\text{S}$, $-\text{SO}_2\text{Cl}$, $-\text{SO}_2\text{CH}=\text{CH}_2$, $-\text{PO}_2\text{Cl}$, $-(\text{CH}_2)_x\text{Hal}$, present at the fluorene ring of the Fmoc or FMS moiety. Examples of these reagents and of the products obtained are depicted in Scheme 7 herein. These derivatized PEGs that can be used according to the invention and similar reagents are commercially available. It should be noted that the PEGylating agent is usually used in its mono-methoxylated form where only one hydroxyl group at one terminus of the PEG molecule is available for conjugation. However, a bifunctional form of PEG where both termini are available for conjugation may be used if, for example, it is desired to obtain a conjugate with two peptide or protein residues covalently attached to a single PEG moiety.

In a preferred embodiment of the invention, the PEG moiety is branched. Branched PEGs are in common use. They can be represented as $\text{R}(\text{PEG}-\text{OH})_m$ in which R represents a central core moiety such as pentaerythritol or glycerol, and m represents the number of branching arms. The number of branching arms (m) can range from three to a hundred or more. The hydroxyl groups are subject to chemical modification.

The use of branched PEG molecules has several advantages including the fact that they require substantially fewer conjugation sites and loss of bioactivity is minimized. Branched PEG molecules are described in US Patents No. 6,113,906, No. 5,919,455, No. 5,643,575, and No. 5,681,567.

The present invention further provides pharmaceutical compositions comprising a pegylated compound according to the invention or a pharmaceutically acceptable salt thereof, and a pharmaceutically acceptable carrier.

The carrier must be "acceptable" in the sense of being compatible with the active ingredient(s) of the formulation (and preferably, capable of stabilizing peptides) and not deleterious to the subject to be treated.

The formulations may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy, for example as described in Remington: The Science and Practice of Pharmacy, A.R. Gennaro, ed., 20th edition, 2000. All methods include the step of bringing the active ingredient(s) into association with the carrier which constitutes one or more accessory ingredients.

Any suitable route of administration of the conjugates of the invention to humans or for veterinary purposes is envisaged by the invention, for example via conventional oral, intramuscular, intravenous, subcutaneous, intranasal and transdermal administration.

The invention further provides methods for treatment of diseases, disorders and conditions treatable by peptide and protein drugs which comprises the administration of a pegylated peptide or protein prodrug of the invention to an individual in need.

The invention will now be illustrated by the following non-limiting Examples.

20

EXAMPLES

I. CHEMICAL SECTION

Materials and Methods (Chemical Section)

(i) *Materials*: Human (Zn^{2+} -free) insulin was donated by Novo Nordisk (Bagsvaerd, Denmark) or by Biotechnology General (Rehovot, Israel), and was used without further purification. Recombinant hGH was a gift from Biotechnology General (Rehovot, Israel). Fmoc-OSu was obtained from Novabiochem (Laüfelfingen, Switzerland). PEG₅-OSu (also referred herein as PEG_{5,000}-OSu) and PEG₄₀-OSu (also referred herein as PEG_{40,000}-OSu) were from Shearwater (now

Nektar Therapeutics, San Carlos, CA, USA). TNBS, DTNB, α -lactalbumin, reduced glutathione (GSH), cystamine-2 HCl, dithiothreitol (DTT), iodoacetamide, gentamicin and TPCK-treated trypsin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Non-glycosylated human IFN- α 2 was prepared as described
5 in WO 02/36067. Exendin-4, Peptide YY₃₋₃₆ and Peptide 27, a nonlysine-containing synthetic peptide of 27 amino acids (AEISGQLSYVRDVNSWQHWTNVSIN) (SEQ ID NO: 14), used as control, were synthesized by the solid phase method using a multiple-peptide synthesizer (AMS 422, Abimed Analysen-Technik GmbH, Langenfeld, Germany). All other reagents, including a long list of compounds used
10 for synthesizing MAL-FMS-NHS, were of analytical grade and purchased from Sigma Chemical Co. (St. Louis, MO, USA).

(ii) *Reverse-phase HPLC* was performed with a Spectra-Physics SP8800 liquid chromatography system (Spectro-Physics, San Jose, CA) equipped with an Applied Biosystem 757 variable wavelength absorbance detector. The column
15 effluents were monitored by UV absorbance at 220 nm and chromatograms were recorded on a chrom-Jet integrator (Thermo-Separation, Riviera Beach, FL, USA). HPLC prepacked columns used in the examples included LiChroCART 250-10 mm containing LiChrosorb RP-18 (7 μ m) and LiChrospher 100 RP-18 (5 μ m) 250-4 mm (Merck, Rathway, NJ, USA) and pre-packed Vydac RP-18 or RP-4 columns
20 (22 x 250 mm; 12 μ m bead size; Vydac, Hesperia, CA, USA). Linear gradients were used between solution A (0.1% TFA in H₂O) and solution B (0.1% TFA in acetonitrile-H₂O, 75:25). For analytical HPLC procedures, a linear gradient between 30 and 100% of solution B was run for 50 min at a flow rate of 0.8 ml/min.

(iii) *HPLC analyses* were performed using a Spectra-Physics SP8800 liquid
25 chromatography system equipped with an Applied Biosystems 757 variable wavelength absorbance detector and a Spectra-SYSTEM P2000 liquid chromatography system equipped with a Spectra-SYSTEM AS100 auto-sampler and a Spectra-SYSTEM UV1000, all controlled by a ThermoQuest chromatography data system (ThermoQuest Inc., San Jose, CA, USA). The column effluents were
30 monitored by UV absorbance at 220 nm. Analytical RP-HPLC was performed using

a prepacked Chromlith™ Performance RP-18e (4.6x100mm, Merck KGaA, Darmstadt, Germany). The column was eluted with a binary gradient of 10-100% solution B over 10 min with a flow rate of 3 ml/min (solution A was 0.1% TFA in H₂O and solution B was 0.1% TFA in acetonitrile:water, 3:1, v:v). Pegylated
5 compounds were analyzed using a RP-4 column (250x4 mm, 5 μm bead size, VYDAC, Hesperia, CA) with a binary gradient of 10-100% solution B in 50 min at a flow rate of 1 ml/min.

(iv) *Mass spectroscopy*: Mass spectra (MS) were determined using matrix-assisted laser-desorption/ionization-time-of-flight (MALDI-TOF) mass
10 spectroscopy (Micromass UK Ltd.) and electrospray ionization mass spectra (ESMS) techniques (Bruker-Reflex-Reflectron model, Germany, and VG-platform-II electrospray single quadrupole mass spectrometer, Micromass UK Ltd., respectively). The polypeptides were deposited on a metal target as cocrystals with sinapic acid, and the mass spectrum was determined in the positive ion mode.

15 (v) *UV spectra*: Ultraviolet spectra were obtained with a Beckman DU 7500 spectrophotometer in 1 cm path length UV cuvettes.

(vi) *Thin-layer chromatography* was performed on silica-gel plates, that were developed either with chloroform:methanol:acetic acid (9.2:0.5:0.3, v:v:v, TLC, A) or by chloroform:methanol (9:1, v:v, TLC, B).

20 (vii) *Amino acid analyses* were performed following 6N HCl acid hydrolysis at 110°C for 24 h using a Dionex Automatic amino acid analyzer HP1090 (Palo Alto, CA, USA). N-terminal sequence analyses were performed with a Model 491A Procise Protein sequencer (Applied Biosystems, Foster City, CA, USA).

Identification of the intermediate compounds - Most of the chemical
25 compounds used as reagents and intermediates in the Examples are identified by their formulas in the Schemes 1-7 hereinafter and the following characterization: the intermediates are identified by a bold underlined letter **a** to **k** (small cap) or by the term Precursor and a number in bold italics, i.e. Precursors *1-8*.

Example 1: Synthesis of PEG_{5,000}-Fmoc-OSu (Precursor 1)

Precursor 1 of the formula depicted in Scheme 1 was prepared starting from 2-aminofluorene and t-Boc-alanine (BocAla) by several steps, as depicted in Scheme 2.

5 1(a). Synthesis of 2-(t-BocAla-amino)fluorene (Intermediate a)

t-Boc-Ala (4.16 gr, 22 mmol) was dissolved in 11 ml dioxane. N,N'-Dicyclohexylcarbodiimide (DCC) (11 mmol in 11 ml 1 M DMF) was then added and the reaction was carried out for 3 hours at 25°C under stirring. Dicyclohexylurea (DCU) formed was removed by centrifugation. The symmetrical
10 anhydride product thus obtained (t-Boc-Ala-anhydride) was reacted overnight under stirring with 2-aminofluorene (0.925 g, 11 mmol) in 30 ml dioxane-water (1:1, v:v) containing 11 mmol of NaHCO₃. The white solid formed was collected and dried under P₂O₅ in vacuum for 24 hours. Intermediate a was obtained in 60% yield (1.31 g, 3.34 mmol). It migrated on TLC (dichloromethane) with R_t=0.17. Mass-
15 spectroscopy revealed a mass of 352.45 Da (calculated mass = 352.2 Da).

1(b). Synthesis of 9-formyl-2-(t-BocAla-amino)fluorene (Intermediate b)

Intermediate a (1.31 g, 3.34 mmol) obtained in Example 1(a) was dissolved in 10 ml dry THF and combined with sodium hydride (NaH, 60%) (0.412 g, 11 mmol, 3.3 equivalents) suspended in dry THF. Ethyl formate (0.675 ml, 8.35 mmol)
20 was then added and the reaction was carried out for 1 hour under stirring and argon atmosphere. After addition of ice chips and water, the organic solvent was removed by evaporation in vacuum. The aqueous solution was washed with ether and acidified to pH 5.0 with acetic acid. The precipitate formed was dissolved in ethyl acetate, the organic solution was washed several times with 0.5 M NaHCO₃, and
25 dried over anhydrous sodium sulfate. The yellow solid formed after evaporation was triturated with ether and dried in vacuum. Intermediate b was obtained in 35% yield (0.46 g, 1.2 mmol). It migrated on TLC (chloroform:methanol:acetic acid, 9.2:0.5:0.3, v:v:v) with R_f=0.59. Mass-spectrum analysis (electrospray ionization technique) revealed a mass of 380.26 Da (calculated mass = 380.2 Da).

1(c). Synthesis of 9-hydroxymethyl-2-(t-BocAla-amino) fluorene (Intermediate c)

Intermediate b (0.46 g, 1.2 mmol) obtained in Example 1(b) was suspended in dry methanol. Solid sodium borohydride (NaBH₄) (57 mg, 1.5 mmol) was added in several aliquots and the reaction mixture was stirred for 4 hours. The crude product obtained was further purified by flash chromatography on silica gel column and eluted with chloroform:methanol (95:5), to yield 80 mg (17%, 0.2 mmol) of pure Intermediate c that migrated on TLC (chloroform:methanol; 9:1, v:v) with R_f=0.53. Mass spectrum analysis revealed a mass of 382.2 Da (calculated mass=382 Da). ¹H-NMR (CD₃SOCD₃)δ: 1.4 (s, 12H), 3.8-4.0 (m, 2H), 4.29 (d, 1H), 6.2 (s, broad), 7.2-7.3 (m, 2H), 7.6-8.0 (m, 4H), 8.4 (s, 1H).

1(d). Synthesis of 9-hydroxymethyl-2-(Ala-amino)fluorene (Intermediate d)

Intermediate c (80 mg, 0.2 mmol) obtained in Example 1(c) was dissolved in 5 ml of dichloromethane:trifluoroacetic acid (TFA) (1:1, v:v). After one hour, the solvents were removed by evaporation, and Intermediate d was suspended in ether, collected by precipitation and lyophilized.

1(e). Synthesis of Intermediate e

To a solution of Intermediate d (0.2 mmol) obtained in Example 1(d) and NaHCO₃ (0.8 mmol) in 3 ml H₂O, PEG_{5,000}-OSu (1 g, 0.2 mmol) was added. The reaction was carried out at 25°C, for several hours, with stirring. Product formation was verified by analytical HPLC procedure using C18 column (R_t=36.6 min). Mass spectrum analysis (MALDI) of Intermediate e revealed a mass of 5342 Da.

1(f). Synthesis of PEG_{5,000}-Fmoc-OSu (Precursor 1)

To a solution of the Intermediate e obtained in Example 1(e) (0.2 mmol) in 2 ml chloroform, triphosgene (1 mmol, 5 molar excess) in 3 ml cold chloroform was added portionwise. Reaction was carried out overnight with stirring. The solvent was then evaporated and the residue was dissolved in 1.0 ml dry THF. N-hydroxysuccinimide (NHS) (100 mg, 4 equivalents) and 2,4,6-trimethylpyridine (0.163 ml, 6 equivalents) were added, and the reaction was carried out for 2 hours at room temperature with stirring. The title product, PEG_{5,000}-Fmoc-OSu, was purified

to homogeneity by preparative HPLC procedure using a C18 column (HPLC, $R_t=39.0$ min). Mass spectrum analysis revealed a mass of 5654 kDa.

Unlike Fmoc-OSu, PEG_{5,000}-Fmoc-OSu is highly soluble in aqueous solutions, and absorbs in the UV region with a molar extinction coefficient of $\epsilon_{280}=21,200$ and $\epsilon_{301}=10,100$.

Example 2: Synthesis of PEG_{5,000}-FMS-OSu (*Precursor 2*)

Precursor 2, PEG_{5,000}-FMS-OSu, depicted in Scheme 1, was prepared by sulfonation of Precursor 1, PEG_{5,000}-Fmoc-OSu, with chlorosulfonic acid, as depicted in Scheme 2. Briefly, to a solution of Precursor 1 in 4.0 ml CH₂Cl₂, cooled to 0°C, a solution of ClSO₃H in CH₂Cl₂ was added dropwise over a period of 15 min and the solution was stirred for 2 hours at 25°C. The product, PEG_{5,000}-FMS-OSu, sulfonated at position 2 of the fluorene ring, was purified by preparative HPLC-procedure, and characterized by mass spectroscopy, elementary analysis (for sulfur), following extensive dialysis, and for its rate of hydrolysis (at pH 8.5, 37°C) following conjugation to either gentamicin or insulin.

Example 3: Synthesis of PEG₄₀₀₀₀-Fmoc-OSu (*Precursor 3*) -

Precursor 3, PEG₄₀₀₀₀-Fmoc-OSu, depicted in Scheme 1, was prepared as described in Example 1, steps 1(e) and 1(f), but replacing PEG₅₀₀₀-OSu with PEG₄₀₀₀₀-OSu under the same reaction conditions.

Example 4: Synthesis of PEG₄₀₀₀₀-FMS-OSu (*Precursor 4*)

Precursor 4, PEG₄₀₀₀₀-FMS-OSu, depicted in Scheme 1, was prepared by sulfonation of Precursor 3, PEG₄₀₀₀₀-Fmoc-OSu, as described in Example 2, but replacing Precursor 1 with Precursor 3 under the same reaction conditions.

Example 5: "One-step" procedure for preparation of PEG-Fmoc/drug conjugates and PEG-FMS-drug conjugates

For the preparation of PEG-Fmoc and PEG-FMS conjugates with drugs

according to the invention, a "one-step" procedure can be used wherein a PEG-Fmoc-OSu or PEG-FMS-OSu precursor reacts with one or more amino groups of the drug in aqueous conditions.

Thus, in the following Examples 6-8, solid Precursor 1, PEG₅₀₀₀-FMS-OSu, obtained in Example 1, was added at a 10-fold molar excess to stirred solutions of gentamicin or insulin (10 mg/ml) in phosphate-buffered saline (PBS) buffer, pH 7.4, at 0°C. Under these conditions, five to seven moles of Precursor 1 were incorporated per mole protein. The reaction was completed within 15 minutes after addition of the solid PEG-Fmoc-OSu.

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Example 6: Synthesis of (PEG₅₀₀₀-Fmoc)₁-gentamicin

Solid Precursor 1 obtained in Example 1 (6 mg, 1 μmol) was added to a stirred solution of gentamicin (200 μmol in 1.0 ml 0.01 M NaHCO₃, pH ~7.5). The reaction was carried out for 3 hours at 25°C and then dialyzed against H₂O at 7°C for several days. Under these dialyzing conditions, free gentamicin, that has not been covalently linked to PEG-Fmoc, was dialyzed out. The product, PEG₅₀₀₀-Fmoc-gentamicin, contained one mol PEG-Fmoc covalently linked to one mol gentamicin as judged by its absorbance at 280 nm, and by amino acid analysis, following acid hydrolysis of a measured aliquot. The hydrolyzate contained alanine (derived from the PEG-ala-Fmoc moiety) and two peaks that emerged at the positions of proline and leucine, following acid hydrolysis of gentamicin (not shown).

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Example 7: Synthesis of (PEG₅₀₀₀-Fmoc)₂-gentamicin

Solid Precursor 1 (11.3 mg, 2.1 μmol) was added to a stirred solution of gentamicin (0.5 mg, 1 μmol, in 1.0 ml 0.05 M NaHCO₃). The reaction was carried out for 2 hours at 25°C and dialyzed overnight. The product, (PEG₅₀₀₀-Fmoc)₂-gentamicin, contained about two moles PEG-Fmoc covalently linked to gentamicin, as verified by amino acid analysis following acid hydrolysis (see Example 6 above) and by determining the amount of non-modified amino groups with TNBS.

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Example 8: Synthesis of (PEG₅₀₀₀-Fmoc)₁-insulin and (PEG₅₀₀₀-Fmoc)₂-insulin

For the preparation of (PEG₅₀₀₀-Fmoc)₁-insulin, to a stirred solution of Zn²⁺-free insulin (1 mg in 2.0 ml 0.01 M NaHCO₃, 0.172 μmol), a fresh solution of Precursor 1 (8.8 μl, 20 mg/ml in DMF) was added (1.76 mg, 0.329 μmol, 1.9 molar excess of reagent over the protein). The reaction was carried out for 2 hours at 25°C. The reaction mixture was dialyzed overnight against H₂O, to remove NaHCO₃ and DMF. The title product contained about one mol PEG-Fmoc per mol insulin, as judged by several procedures described in the Biological Section hereinafter. The concentration of insulin in the sample was routinely determined by acid hydrolysis of a 20 μl aliquot, followed by amino acid analysis, and was calculated according to glutamic acid (7 residues), aspartic acid (3 residues) and isoleucine (2 residues).

The preparation of (PEG₅₀₀₀-Fmoc)₂-insulin was carried out as described above while using 4.63 mg, 0.865 μmol, 5 molar excess of reagent over the protein.

Example 9: Synthesis of N-[2-(3-acetylthiopropionylamino)-9-fluorenylmethoxycarbonyloxy]-succinimide (Precursor 5)

Precursor 5, depicted in Scheme 3, was synthesized starting from 2-aminofluorene by the procedure depicted in Scheme 4, as follows:

9(a). Synthesis of 2-(*t*-Boc-amino)fluorene (Intermediate *f*)

Di-*t*-butyl-dicarbonate (Boc anhydride, 14 g, 64.2 mmol) solution in dioxane (50 ml) was combined with 2-aminofluorene (10 g, 55 mmol in 100 ml dioxane:water, 1:1, v:v) and with NaHCO₃ (9.24 g, 110 mmol), and stirred overnight. The white solid thus formed was filtered, washed with ice water (200 ml) and dried under high vacuum. Intermediate *f* was obtained in 60% yield (9.24 g, 33 mmol). TLC (chloroform:methanol:acetic acid, 9.5:0.5:0.3, v:v:v); R_f=0.73. Calculated ESMS=280.4 Da, found ESMS=280.45 Da.

9(b). Synthesis of 9-formyl-2-(*t*-Boc-amino)fluorene (Intermediate *g*)

Intermediate *f* (3 g, 10 mmol), obtained in step 9(a), was dissolved in dry THF (30 ml) and added to a suspension of sodium hydride (NaH) (60%, 1.23 g, 33 mmol, 3.3 eq) in dry THF under Argon atmosphere. Ethyl formate (2 ml, 25 mmol, 2.5 eq) was then added and the reaction mixture was stirred for 1 hour. Ice chips and water were added, the organic solvent was evaporated, and the aqueous solution was washed with ether and acidified with acetic acid (pH ~5). The precipitate thus formed was dissolved in ethyl acetate, washed with NaHCO₃ (0.5 N), brine and dried over anhydrous sodium sulfate. The yellow solid was washed with ether and dried. Intermediate *g* was obtained in 90% yield (2.8 g, 9 mmol). TLC (chloroform: methanol:acetic acid; 9.5:0.5:0.3, v:v:v), R_f=0.66. Calculated ESMS=309 Da, found ESMS=309.2 Da. M-1: 308.20, M+Na: 332.36, dimer [M+Na]⁺: 641.59.

9(c) Synthesis of 9-hydroxymethyl-2-(t-Boc-amino)fluorene (Intermediate h)

Sodium borohydride (NaBH₄) (0.38 g, 10 mmol) was added portionwise to a suspension of Intermediate *g* (2.8 g, 9 mmol) obtained in step 9(b), in dry methanol, and the reaction allowed to proceed for 4 hours. The product, Intermediate *h*, was purified by flash chromatography on silica gel column, that was eluted with chloroform:methanol (98:2, v:v), and was obtained in 50% yield (1.4 g, 4.5 mmol). TLC (chloroform:methanol, 9:1, v:v), R_f = 0.54; Calculated ESMS=311 Da, found ESMS=311.42 Da, for [M+Na]⁺ = 334.42, for [M+K]⁺ = 350.34.

9(d). Synthesis of 9-hydroxymethyl-2-aminofluorene (Intermediate i)

Intermediate *h* (1.4 g, 4.5 mmol) obtained in step 9(c) was dissolved in 110 ml 5N HCl in dioxane. After 1 hour, the solvent was concentrated by evaporation and the product was precipitated with ether and lyophilized. Intermediate *i* was obtained in 84% yield (0.79 g, 3.78 mmol). TLC (chloroform:methanol, 9:1, v:v), R_f = 0.38; calculated ESMS=211.26 Da, found ESMS for [M+H]⁺ = 211.10 Da.

9(e). Synthesis of 3-S-acetylthiopropionic acid

Pyridine (6.9 ml, 84.6 mmol) was added to a mixture of 3-mercaptopropionic acid (2.5 ml, 28.2 mmol) and acetic anhydride (16 ml, 84.6 mmol). The reaction solution was stirred for 16 h and concentrated by vacuum. Water (5 ml) was added for 20 min and the solution was concentrated by vacuum. The obtained oil was

dissolved in ether (50 ml) and washed with water and KHSO₄ (0.5 N). The ether fraction was dried with Na₂SO₄ and vacuum to produce the title product (Yield: 85%, 24 mmol, 3.6 g). ¹H-NMR (CDCl₃)δ: 2.21 (s, 3H), 2.6 (t, 2H), 2.9 (t, 2H).

9(f). Synthesis of 3-S-acetylthiopropionic anhydride

5 3-S-acetylthiopropionic acid of step 9(e) (1.8 g, 12 mmol) and DCC (1.4 g, 6 mmol) were dissolved in DMF (15 ml) for 4 hours. The precipitated DCU was filtered out and the anhydride thus formed was kept at 4°C until used.

9(g). Synthesis of 9-hydroxymethyl-2-(3-acetylthiopropionyl-amino)-fluorene (Intermediate j)

10 Intermediate *i* (0.422 g, 2 mmol) obtained in step 9(d) and NaHCO₃ (0.74 g, 18 mmol) were dissolved in water/dioxane (1:1, 20 ml) and 3-S-acetylthiopropionic anhydride of step 9(f) in DMF (17 ml, 6 mmol) was added. The reaction solution was stirred for 1 hour. The organic solvents were removed by vacuum and the liquid solution was extracted with ether, washed with Na₂SO₄ (0.5 N) and water and dried
15 by vacuum. The obtained crude product, Intermediate *j*, was further purified by flash chromatography on silica gel column and elution with ethyl acetate:hexane (1:1, v:v). Yield: 60%, 0.4 g, 1.2 mmol. ESMS, (ca. 431) M+Na⁺: 464, dimer 2M: 682, dimer 2M: Na⁺: 705.67. ¹H-NMR (CDCl₃)δ: HPLC (Chromolith column) 5.3 min (10-100% solution B in 10 min, 3 ml/min).

20 *9(h). Synthesis of Precursor 5*

Intermediate *j* obtained in step 9(g) was reacted with N-hydroxysuccinimide and phosgene, as depicted in Scheme 4. Thus, pyridine (0.215 ml, 2.7 mmol) was added dropwise to a stirred solution of Intermediate *j* (0.385 g, 0.9 mmol) and triphosgene (0.265 g, 0.9 mmol, 3 eq) in dry THF (5 ml). After 20 min, the
25 precipitated pyridine hydrochloride salt was filtered out and the THF was removed by evaporation. The obtained oil was dissolved in dry THF (10 ml). N-hydroxysuccinimide (0.5 g, 4.4 mmol, 5 eq) and pyridine (0.215 ml, 2.7 mmol) were then added and the solution stirred for 20 min. The precipitated pyridine hydrochloride salt was filtered out and the THF was removed by vacuum. The
30 obtained oil was further purified by flash chromatography on silica gel column and

elution with ethyl acetate:hexane (1:1 and 4:1). The product, Precursor 5, was obtained in a yield of 88% (0.38g, 0.78 mmol). ESMS: (ca 482) M: 482.2. HPLC (Chromolith column) 6.68 min (10-100% solution B in 10 min, 3 ml/min). TLC (ethyl acetate:hexane, 1:1, v:v) Rf=0.4.

5

Example 10: Synthesis of N-[2-(3-acetylthiopropionylamino)-7-sulfo-9-fluorenylmethoxycarbonyloxy]-succinimide (Precursor 6)

Precursor 6, depicted in Scheme 3, was prepared by sulfonation of Precursor 5 with chlorosulfonic acid as described above in Example 2.

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Example 11: Synthesis of MAL-Fmoc-NHS, N-[2-(maleimidopropionylamino)fluoren-9-yl-methoxycarbonyloxy[succinimide)] (Precursor 7)

Precursor 7, MAL-Fmoc-NHS depicted in Scheme 3, was prepared as depicted in Scheme 5 starting from 2-aminofluorene through the synthesis of the Intermediates *f* to *i*, described in Example 9 above, followed by the steps below:

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11(a). Synthesis of 9-Hydroxymethyl-2-(maleimidopropionylamino)fluorene (Intermediate *k*)

3-Maleimidopropionic anhydride was prepared by dissolving 3-maleimidopropionic acid (1 g, 5.9 mmol) and DCC (0.69 g, 2.95 mmol) in 10 ml DMF and incubating for 4 h. DCU was filtered out and the anhydride thus formed was kept at 4°C.

20

9-Hydroxymethyl-2-amino-fluorene (Intermediate *i*; (0.4 g, 1.9 mmol) and NaHCO₃ (0.74 g, 8.85 mmol) were dissolved in water and 3-maleimidopropionic anhydride in DMF (12 ml, 2.95 mmol) was added. The reaction mixture was stirred for 40 min and product formation monitored by analytical HPLC on a Chromolith column (Rt=4.46 min, 10-100% B in 10 min, 3 ml/min). The crude product, Intermediate *k*, was purified using preparative HPLC (RP-18 column, 10-100% acetonitril:water [75:25; v:v], 60 min, 12 ml/min). Yield: 57%, 1.08 mmol, 0.39 g. Calculated ESMS = 362.38 Da, found ESMS = 362.42 Da).

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11(b). Synthesis of MAL-FMOC-NHS

Pyridine (0.167 ml, 2 mmol) was added dropwise to a stirred solution of Intermediate *k* (0.37 g, 1.02 mmol) obtained in step 11(a) and triphosgene (0.425 g, 1.43 mmol, 4.2 eq) in dry THF (10 ml). After 20 min the precipitated pyridine hydrochloride salt was filtered out and the THF removed by vacuum. The oil obtained was dissolved in 10 ml dry THF with N-hydroxysuccinimide (0.61g, 5.3 mmol). Pyridine (0.26 ml, 3.2 mmol) was then added and the solution was stirred for 20 min. Some additional precipitated pyridine hydrochloride salt was filtered out and the THF removed by vacuum. The oil obtained was dissolved in chloroform (100 ml) and washed with an aqueous NaHCO₃ solution (0.5 N, 3× 50 ml), HCl (0.1 N, 3× 50 ml), water (2× 50 ml) and brine. The chloroform was removed by vacuum and the product, Precursor 7, was desiccated. Yield: 89% (0.9 mmol, 0.45 g). HPLC (Chromolith column) Rt=5.7 min (10-100% B in 10 min, 3 ml/min). Calculated ESMS = 503 Da, found ESMS for [M+Na]⁺ = 526.38 Da, found ESMS for [M+K]⁺ = 542.30 Da.

Example 12: Synthesis of MAL-FMS-NHS, N-[2-(maleimido-propionylamino)-7-sulfo-fluoren-9-yl]methoxycarbonyloxysuccinimide (Precursor 8)

Precursor 8, depicted in Scheme 3, herein also identified by the abbreviation MAL-FMS-NHS (or MAL-FMS-OSu), was prepared by sulfonation of Precursor 7, MAL-Fmoc-NHS, with chlorosulfonic acid as depicted in Scheme 5, as follows:

To a solution of Precursor 7 (0.2 mmol, 0.1 g) obtained in Example 11 in trifluoroacetic acid (TFA) (10 ml), chlorosulfonic acid (0.5 ml) was added. After 15 minutes, cold ether (90 ml) was added and the precipitated product. Precursor 8, was washed with ether (×3) and desiccated. Yield: 95% (0.11 g, 0.19 mmol). HPLC (Chromolith column) Rt=2.65 min (10-100% B in 10 min, 3 ml/min). Calculated ESMS = 583 Da, found ESMS for [M-H]⁺ = 582.24 Da, for [M+H]⁺ = 584.52 Da, and for [M+Na]⁺ = 606.47 Da.

Example 13: Chemical characterization of MAL-FMS-NHS

MAL-FMS-NHS or MAL-FMS-OSu is a water-soluble, hetero-bifunctional reagent consisting of a sulfonated fluorenylmethoxycarbonyl N-hydroxy-succinimide ester that reacts with peptide and protein amino groups. A maleimide group was attached to the fluorenyl backbone to enable coupling to sulfhydryl-containing PEG₄₀₀₀₀-SH.

MAL-FMS-NHS is a cleavable reagent capable of reacting covalently with the amino side chains of peptides, proteins and aminoglycosides. According to the present invention, it enables PEG chains to be linked to peptides and proteins through a slowly hydrolysable chemical bond. PEG-FMS-peptide/protein conjugates thus formed undergo spontaneous hydrolysis at a slow rate upon incubation at pH 8.5, 37°C, with a $t_{1/2}$ value of $8-14 \pm 2$ h, generating the unmodified parent molecule.

Chemical features of MAL-FMS-NHS. Table 1 summarizes several of the characteristic features of MAL-FMS-NHS. It is a water- and DMF-soluble compound. Mass spectrum analysis has yielded a calculated mass of 583 Da. The compound absorbs in the U.V. region with a molar extinction coefficient $\epsilon_{280}=21,000\pm 200 \text{ mol}^{-1}\text{cm}^{-1}$. The curve shows a maximum at 290 nm, and a shoulder extending up to 330 nm. At 320 nm, where peptides or proteins absorb negligibly, MAL-FMS-NHS, either free or covalently bound to proteins, absorbs with a molar extinction coefficient $\epsilon_{320}=16,100\pm 150 \text{ mol}^{-1}\text{cm}^{-1}$ (Table 1).

Stability of the MAL-functional moiety in aqueous solutions. In order to test the stability of the maleimide function of MAL-FMS-NHS in aqueous solutions at different pH values, MAL-FMS-NHS (1 mM) was incubated at room temperature in water (pH 6.0), in 0.007M acetic acid (pH~4.0), in 0.1M phosphate buffer (pH 7.4), and in 0.1M NaHCO₃ (pH 8.5). At several time points, aliquots were allowed to react with a slight excess of reduced glutathione (GSH) (15 min at pH 7.2) and the concentration of unreacted GSH was determined with DTNB (5,5'-dithio-bis-2-nitrobenzoic acid, Ellman's reagent), by measuring the absorbance of the produced

yellow colored 5-thio-2-nitrobenzoic acid (TNB) at 405 or 412 nm. Fig. 1 demonstrates the potency of the reaction of MAL-FMS-NHS with GSH as a function of time, at different pH values. MAL-FMS-NHS was found fully stable at pH 4 to 6 over a period of a day or two and at pH 7.2 it is stable over a period of several hours. At pH 8.5, the maleimide moiety was destroyed at an accelerated rate ($t_{1/2}$ value = 2.7 ± 0.3 hr, Fig. 1).

Example 14: Degree of incorporation of MAL-FMS-NHS at pH 7.2 into α -lactalbumin as a function of the amount of added reagent

To evaluate the degree of incorporation of the maleimide moiety into peptides/proteins as a function of the reagent, α -lactalbumin was used as a model. Thus, to samples of α -lactalbumin (α -LA, 1.0 ml of 1mg/ml), MAL-FMS-NHS was added in 0.1M PBS, pH 7.2, at concentrations ranging from 1 equivalent up to 14 molar equivalents of MAL-FMS-NHS, for 30 min at 25°C, that is, under experimental conditions where the maleimide-alkylating capacity of the spacer remains unmodified. For each treatment, the amount incorporated into the protein was determined by UV absorbance at 280 nm, after dialysis, and by quantitating the amount of unmodified amino side-chain moieties with TNBS. As shown in Fig. 2, about one mole of MAL-FMS was incorporated into α -LA per 2.1 mole of reagent added, up to a fourteen molar excess over the protein.

Example 15: Synthesis of PEG₄₀-SH

PEG₄₀-SH was prepared either by reaction of 2-tritylthioethylamine and PEG₄₀-OSu as described below in 15(a), or from PEG₄₀-OSu and cystamine [2,2'-dithio-bis(ethylamine)] dihydrochloride, as described below in 15 (b).

15(a). Synthesis of PEG₄₀-SH from 2-tritylthioethylamine and PEG₄₀-OSu

Synthesis of 2-tritylthioethylamine. 2-Amino-ethanethiol (3 g, 26.5 mmol) and triphenylmethyl alcohol (7 g, 26.9 mmol) were dissolved in TFA (20 ml) and stirred for 30 min at room temperature. The TFA was removed by evaporation and the remaining oil residue was dissolved in ether (400 ml) and stored at -20°C. The

product isolated was washed with cooled ether and water. A concentrated ammonia solution (25%, 30 ml) was then added, and the aqueous phase was washed several times with ether. The ether was removed by vacuum and the product was desiccated. Yield, 78% (6.6 g, 20.7 mmol), $R_t=5.35$ min.

5 2-Tritylthioethylamine and PEG₄₀-OSu (0.25 g, 6.25 μ mol) were dissolved in 4 ml THF:acetonitrile (1:2, v:v). After one hour, the product was precipitated with cooled ether and washed ($\times 3$). The dry pellet was dissolved in TFA:triethylsilane:water (95:2.5:2.5, 6 ml) and, after one hour, PEG₄₀-SH was precipitated by cooled ether (100 ml) and desiccated.

10 ***15(b). Synthesis of PEG₄₀-SH from cystamine and PEG₄₀-OSu***

PEG₄₀-OSu was dissolved at a concentration of 40 mg/ml in an aqueous solution of cystamine-di-HCl (1M) and brought to pH 8.5 with NaHCO₃. Reaction was carried out for 2 h at 25°C. The product thus obtained was dialyzed overnight against 0.1 M NaHCO₃, treated with 30 mM dithiothreitol (25°C, 1 h) and re-
15 dialyzed against 0.01 M HCl containing 10 mM ascorbic acid. PEG₄₀-SH was obtained with a 93% yield. It contained 1 mole sulfhydryl moiety per mole PEG₄₀, as determined with DTNB in the presence of ascorbate. PEG₄₀-SH was kept frozen until used.

20 **Example 16: Preparation of PEG-FMS-drug conjugates from MAL-FMS-NHS (Procedure I)**

For the preparation of PEG-FMS-drug conjugates according to the invention, a “two-step” Procedure I can be used as depicted in Scheme 6 whereby MAL-FMS-NHS is first reacted with an amino-containing drug or with the amino group of a
25 peptide or protein drug and the resulting MAL-FMS-drug conjugate is further reacted with PEG-SH. as follows:

16(a). Preparation of MAL-FMS-drug conjugates

In the first step, a MAL-FMS-drug conjugate is prepared by adding one to seven molar excess of MAL-FMS-NHS, Precursor 8, to a stirred solution of the
30 drug (1 mg/ml) in 0.1 M PBS buffer, pH 7.2. The reaction is carried out for 30 min

at 25°C, and then dialyzed against water at 7°C over a period of 24 h to remove excess MAL-FMS-NHS. The amount of FMS-MAL residues incorporated into the drug, e.g. a peptide or protein drug, is determined both by the absorption at 320 nm using molar extinction coefficient of $\epsilon_{320}=16,100$, and by quantitating the incorporated MAL function into the drug. This is performed by adding a measured aliquot into a GSH solution (0.1 mM in phosphate buffer, pH 7.2). The concentration of unreacted GSH remained is determined with DTNB.

16(b). Preparation of PEG-FMS-drug conjugates

In the second step, a stoichiometric amount of solid PEG₄₀-SH is added to the MAL-FMS-drug conjugate of step 16(a), and the reaction proceeds for additional 60 min in 0.1 M phosphate buffer, pH 7.2, containing 2 mM ascorbic acid. The PEG-FMS-drug conjugate thus obtained is further purified by HPLC-procedures using C4 or C18 reverse phase columns under conditions resolving the conjugate from the drug, preferably a peptide or protein drug (or protein-FMS-MAL) that has not been covalently linked to PEG₄₀-SH.

Example 17: Preparation of PEG-FMS-drug conjugates from MAL-FMS-NHS (Procedure II)

PEG-FMS-drug conjugates according to the invention may be also prepared by an alternative “two-step” Procedure II depicted in Scheme 6 whereby MAL-FMS-NHS is first reacted with PEG-SH and the resulting PEG-FMS-NHS conjugate is further reacted with an amino-containing drug or with the amino group of a peptide or protein drug. as follows:

17(a). Preparation of PEG-FMS-NHS conjugate

In the first step, a PEG-FMS-NHS conjugate is prepared by MAL-FMS-NHS to a stirred solution of PEG-SH in PBS buffer, pH 7.4. The reaction is carried out for 30 min at 25°C and the product is then purified by HPLC and lyophilized.

17(b). Preparation of PEG-FMS-drug conjugates

In the second step, a stoichiometric amount of the MAL-FMS-NHS conjugate of step 17(a) is added to the amino-containing drug, and the reaction

proceeds under stirring for 2 hours. The product is then purified by HPLC and lyophilized.

Example 18: Synthesis of (PEG₄₀-FMS)₁-insulin.

5 (PEG₄₀-FMS)₁-insulin, was prepared by the two-step Procedure I described in Example 16. Thus, Mal-FMS-NHS (6 g, 10 μ mol) was dissolved in water (0.25 ml) and added to an insulin (Zn²⁺-free, 25 mg, 4.16 μ mol) solution in PBS (0.5 ml, pH=7.4). The pH was adjusted to 7-8 with NaHCO₃. The reaction was stopped after 30 min with diluted hydrochloric solution (pH=6). The product was isolated using
10 preparative HPLC (C18 column, 10-80% acetonitrile:water, 75:25, v:v, 50 min, 10 ml/min) and identified as monosubstituted insulin derivative Mal-FMS-insulin using electrospray mass spectrometry (ESMS (ca. 6280), M-1: 6278.83, M+1: 6280.73). Analytical HPLC (Chromolith column), Rt=4.58 min.

PEG₄₀-SH (20 mg, 0.5 μ mol) was added to a solution of the Mal-FMS-
15 insulin intermediate (2 mg, 0.317 μ mol) in PBS (0.3 ml, pH=7.4), the pH was adjusted to 7-8 with NaHCO₃ and the reaction was stopped after 30 min with diluted hydrochloric solution (pH=6). The title product, PEG₄₀-FMS-insulin, was subjected to analytical HPLC (Chromolith column), Rt= 6.6 min.

20 **Example 19: Synthesis of PEG₄₀-FMS-exendin-4.**

PEG₄₀-FMS-exendin-4 was prepared by the two-step Procedure I described in Example 16. Thus, 280 μ g of MAL-FMS-NHS (28 μ l from a fresh solution of MAL-FMS-NHS in DMF, 10 mg/ml, 2.0 molar excess over the peptide) was added to a stirred solution of exendin-4 (1 mg in 1.0 ml 0.1 M phosphate buffer, 2 mM
25 ascorbate, pH 7.2, 0.239 mM). After 7 min, solid PEG₄₀₀₀₀-SH was added to a final concentration of 0.5 mM (2.1 molar excess over the peptide). The reaction was carried out for 1 h hour, dialyzed overnight against water and then further filtered through Centricon with a molecular weight cutoff of 50 kDa to remove any residual exendin-4 or MAL-FMS-exendin-4 that had not linked to PEG₄₀₀₀₀-SH. The
30 concentration of the conjugate was determined by acid hydrolysis of a 20 μ l aliquot

followed by amino acid analysis, according to aspartic acid (2 residues), alanine (2 residues) and valine (1 residue), and absorbance at 280 nm was monitored. The calculated extinction coefficient at 280 nm is 26940 ± 100 , a value that is the additive absorbance of exendin-4 ($\epsilon_{280} = 5740$) and PEG₄₀₀₀₀-FMS ($\epsilon_{280} = 21200$). A solution of 20 μM PEG₄₀₀₀₀-FMS-exendin had $\text{OD}_{280} = 0.51$. Thus the conjugate had a 1:1 PEG₄₀₀₀₀-FMS/exendin stoichiometry (as determined from the UV absorption of the conjugate following purification and acid hydrolysis followed by amino acid analysis as described above).

10 **Example 20: Synthesis of PEG₅₀₀₀-FMS-exendin-4.**

PEG₅₀₀₀-FMS-exendin-4 was prepared by the two-step Procedure II described in Example 17. Thus, MAL-FMS-MHS (1.8 mg, 3 μmol) was added to a solution of PEG₅₀₀₀-SH (15 mg, $\sim 3 \mu\text{mol}$) in PBS (0.5 ml, pH=7.4) and the reaction solution was stirred for 30 min. The product PEG₅₀₀₀-FMS-NHS was purified by HPLC on a RP-4 column and lyophilized.

PEG₅₀₀₀-FMS-NHS (2.3 mg, $\sim 0.42 \mu\text{mol}$) was then added to a solution of exendin-4 (1.6 mg, 0.4 μmol) in PBS (0.5 ml, pH=8) and the reaction solution was stirred for 2 h. The product PEG₅₀₀₀-FMS-exendin-4 was purified by HPLC on a RP-4 column and lyophilized.

20

Example 21: Synthesis and characterization of PEG₄₀-FMS-IFN α 2

The attachment of a single PEG-chain of 40 kDa to IFN α 2 appears sufficient to grossly arrest kidney filtration of the conjugate (Bailon et al., 2001). We therefore envisioned that the linkage of a single PEG₄₀-FMS chain to IFN α 2 would suffice to obtain a prolonged-acting conjugate that releases IFN α 2, with a desirable pharmacokinetic profile. In the procedure found most optimal for introducing one mole PEG₄₀-FMS/mol protein, IFN α 2 was allowed to react first with 3 equivalents of MAL-FMS-NHS, at pH 7.2, for 7 min, followed by the addition of 3 equivalents of PEG₄₀-SH, at pH 7.2, for 1 hour. The NHS function of MAL-FMS-NHS is

relatively unstable at prolonged aqueous neutral conditions, whereas the MAL function of the spacer preserves its alkylating capacity for several hours at pH 7.2 (not shown). We therefore preferred to use Procedure I of Example 16 and to react MAL-FMS-NHS first with IFN α 2 and to subsequently link PEG₄₀-SH to the
5 IFN α 2-FMS-MAL conjugate.

To a stirred solution of IFN α 2 (1 mg/1.0 ml) in phosphate buffer, pH 7.2 (52 μ M), 91 μ g of MAL-FMS-NHS was added (9.1 μ l from a fresh solution of MAL-FMS-NHS, (10 mg/ml) in DMF, (3.0 molar excess over the protein). After 7 min, PEG₄₀-SH was added to obtain a final concentration of 156 μ M (three molar excess
10 over the protein). The reaction was carried out for 1 h, and then dialyzed overnight against H₂O to remove residual DMF and phosphate buffer. The conjugate thus obtained was characterized by MALDI-TOF as PEG₄₀-FMS-IFN- α 2.

Table 2 summarizes several characteristic features of the conjugate thus obtained. MALDI-TOF mass spectra analysis shows a 1:1 PEG₄₀-FMS/IFN α 2
15 stoichiometry. The experimental mass obtained, 63540 Da, corresponds to the additive masses found for PEG₄₀-SH (43818 Da), IFN α 2 (19278 Da) and of the spacer molecule following conjugation (473 Da). PEG₄₀-FMS-IFN α 2 migrates on analytical HPLC as a wide peak with Rt value = 43 min. The conjugate is highly soluble in aqueous solutions. It has a molar extinction coefficient $\epsilon_{280} = 39270 \pm 100$,
20 corresponding to the absorption of the native cytokine and of FMS ($\epsilon_{280} = 21,200$) (Gershonov et al., 2000).

Example 22: Synthesis of (PEG₄₀-FMS)₂-IFN α 2.

(PEG₄₀-FMS)₂-IFN α 2 was prepared as described in Example 21 above for
25 PEG₄₀-FMS-IFN α 2, but using 6 eq (16 μ g, 44.5 nmol, in 10 μ l DMF) of MAL-FMS-NHS.

Example 23: Synthesis of PEG₄₀-FMS-PYY₃₋₃₆

In the procedure found most optimal for coupling about one mole of PEG₄₀-SH per mol of MAL-FMS-PYY₃₋₃₆, PYY₃₋₃₆ was allowed to react first with 2 equivalents of MAL-FMS-NHS, at pH 7.2, for 7 min, followed by the addition of 2.1 equivalents of PEG₄₀-SH, at pH 7.2, for 1 hour.

To a stirred solution of PYY₃₋₃₆ (1 mg in 1.0 ml phosphate buffer, pH 7.2, 10 mM Na ascorbate (0.247 μM), 288 μg of MAL-FMS-NHS was added (28.8 μl, from a fresh 10 mg/ml solution of MAL-FMS-NHS in DMF; twofold molar excess over the peptide). After 7 min, PEG₄₀-SH was added to obtain a final concentration of 0.52 mM (2.1 molar excess over the peptide). The reaction was carried out for 1 h and the mixture was then dialyzed overnight against water. The resulting PEG₄₀-FMS-PYY₃₋₃₆ was characterized by MALDI-TOF MS.

Example 24: Synthesis of (PEG₄₀-FMS)₂-hGH

(PEG₄₀-FMS)₂-hGH was prepared by the two-step procedure described in Example 16, as follows: To a stirred solution of human growth hormone (4.4 mg, 0.22 μmol) in phosphate buffer (1 ml, 0.1 M, pH 7.2), MAL-FMS-NHS (4 eq, 0.47 mg, 0.8 μmol) was added. The reaction was carried out for 30 min at 25°C, and then dialyzed against H₂O (pH 6) at 4°C for 24 h, to remove excess MAL-FMS-NHS. Solid PEG₄₀-SH (7 eq, 65 mg, 1.5 μmol) was added, and the reaction proceeded for 2 h in phosphate buffer (1 ml, 0.1M, pH 7.2, containing 2 mM ascorbic acid). The thus obtained title compound was purified by RP-HPLC using a C4 column, and characterized by SDS gel electrophoresis (12.5 %) indicating the presence of (PEG₄₀-FMS)₂-hGH.

Example 25: Synthesis of PEG₄₀-FMS-hGH

MAL-FMS-NHS (280 μg) was added to a solution of hGH (4.5 mg, 0.225 μmol) in 0.5 ml PBS, pH=7.4. After 10 min, PEG₄₀-SH (10 mg) was added and the reaction mixture was stirred for 1 h and dialyzed overnight against water. The product, PEG₄₀-FMS-hGH, was purified by RP-HPLC.

Example 26: Synthesis of PEG₄₀-FMS-ANP

To a stirred solution of atrial natriuretic peptide (ANP) (0.32 μmol , 1 mg) in phosphate buffer (1 ml, 0.1 M, pH 7.2), MAL-FMS-NHS (2 eq, 0.2 mg, 0.7 μmol) was added. The reaction was carried out for 10 min at 25°C. Solid PEG₄₀-SH (2.2 eq, 30 mg, 0.7 μmol) was added, and the reaction proceeded for 2 h in phosphate buffer (1 ml, 0.1M, pH 7.2, containing 2 mM ascorbic acid). The product was further purified by HPLC.

10 II. BIOLOGICAL SECTION

In this section, the biological activity of the PEG-Fmoc and PEG-FMS conjugates with gentamicin, peptides and proteins prepared in the examples above, was tested.

15 Example 27: Inactive PEG-Fmoc-gentamicin conjugates undergo reactivation

For assaying the antibacterial potency of gentamicin and its derivatives, a suspension of *Escherichia coli* (*E. coli* strain N-4156-W.T, 1% v/v in LB medium) was divided into plastic tubes (0.5 ml per tube) and incubated in a shaking water bath at 37°C, in either the absence or the presence of increasing concentrations of gentamicin (0.02 to 2 μM), and increasing concentrations of PEG_{5,000}-gentamicin derivatives (0.02-50 μM). *E. coli* replication was evaluated by measuring the absorbance at 600 nm. Incubation was terminated when O.D₆₀₀ nm in the tubes containing no gentamicin reached a value of 0.6 ± 0.1 . Under our assay conditions, native gentamicin inhibited half-maximally *E. coli* replication at a concentration of 0.22 ± 0.02 μM (0.1 $\mu\text{g/ml}$). A PEG-gentamicin derivative showing an IC₅₀ value of 2.2 ± 0.2 μM in this assay is considered having 10% the antibacterial potency of native gentamicin.

(PEG_{5,000}-Fmoc)₁-gentamicin and (PEG_{5,000}-Fmoc)₂-gentamicin, containing one or two moles of PEG_{5,000}-Fmoc/mol gentamicin, respectively, were prepared as a model in order to assess the reversibility of the PEG-Fmoc moieties. Both

derivatives (0.2 mM of each) were incubated in 0.1 M NaHCO₃ (pH 8.5) at 37°C, and aliquots were withdrawn at different times and then analyzed for their potency to arrest *E. coli* replication (see method (xi) above). The IC₅₀ for each aliquot was determined. Native gentamicin inhibited *E. coli* replication with IC₅₀ value of 0.22±0.2 μM. (PEG_{5,000}-Fmoc)₂- and (PEG_{5,000}-Fmoc)₁-gentamicin had 0.01 and 2.1±0.1% of the antibacterial potency of gentamicin, respectively.

The results are shown in Figs. 3A-3B. (PEG₅₀₀₀-Fmoc)₂-gentamicin showed a prolonged lag period (about 15 hours) prior to noticeable reactivation (Fig. 3A). A sharp elevation in antibacterial potency then occurred, reaching 50 or 100% of the native potency after 30±2 or 60±3 hours of incubation, respectively (Fig. 3A). No lag period was observed upon incubation of (PEG_{5,000}-Fmoc)₁-gentamicin. Reactivation proceeded continuously with half-maximal reactivation at 6±0.3 hours, and full activation (100%) after 30 hours of incubation (Fig. 3B).

15 Example 28. Biological activity of PEG-Fmoc-insulin conjugates

Materials and Methods

(i) *Materials.* Recombinant human insulin was from Biotechnology General (Rehovot, Israel). D-[U-¹⁴C]Glucose (4-7 mCi/mol) was obtained from Du Pont NEN (Boston, MA, USA). Collagenase type I (134 U/mg) was purchased from 20 Worthington (Freehold, NJ, USA).

(ii) *Rat adipocytes* were prepared from fat pads of male Wistar rats (100-200 gr) by collagenase digestion as described (Rodbell, 1964). The fat pads were cut into small pieces with scissors and suspended in 3 ml of KRB buffer containing NaCl, 110 mM; NaHCO₃, 25 mM; KCl, 5 mM; KH₂PO₄, 1.2 mM; CaCl₂, 1.3 mM; 25 MgSO₄, 1.3 mM; and 0.7% BSA (pH 7.4). Digestion was performed with collagenase type I (1 mg/ml) in a 25 ml flexible plastic bottle under an atmosphere of carbogen (95% O₂, 5% CO₂) for 40 min at 37°C with vigorous shaking. Five ml of buffer was then added, and the cells were passed through a mesh screen. The cells were then allowed to stand for several minutes in a 15 ml plastic test tube at

room temperature, floating, and the buffer underneath was removed. This procedure (suspension, floating, and removal of buffer underneath) was repeated three times.

(iii) *Lipogenesis (incorporation of [U-¹⁴C] glucose into the lipids of intact adipocytes)*. The incorporation of [U-¹⁴C] glucose into adipose tissue in rat adipocytes was performed as described (Moody et al., 1974). Adipocyte suspensions (3×10^5 cells/ml) were divided into plastic vials (0.5 ml per vial) and incubated for 60 min at 37°C under an atmosphere of carbogen with 0.2 mM [U-¹⁴C]glucose, in either the absence or presence of insulin. Lipogenesis was terminated by adding toluene-based scintillation fluid (1.0 ml per vial) and the radioactivity in extracted lipids was counted. In a typical experiment, insulin-stimulated lipogenesis was 4-5 fold higher than basal (basal: 2000 cpm per 3×10^5 cell/h; V_{insulin} 8,000-10,000 cpm per 3×10^5 cells/h). In this assay, insulin stimulates lipogenesis with ED₅₀ value = 0.15 ± 0.03 ng/ml. An insulin analog exhibiting ED₅₀ value = 15 ng/ml is considered to have ~1 % of the biological potency of the native hormone.

(iv) *Glucose-lowering potency of insulin and its derivatives* was determined in mice following administration under the conditions specified in each experiment. Blood samples were taken from the tail vein at different time points after administration, and blood glucose levels were determined with glucose analyzer (Beckman Instrumen, Fullerton, CA, USA) by the glucose oxidase method. The level of glucose in normal healthy CD1-mice was 140 ± 7 mg/dl (7.77 mM). Each group consisted of 4-5 mice. Data are presented as means \pm SEM.

28 (i). *Progressive modification of amino acid moieties of insulin with PEG₅₀₀₀-Fmoc-OSu.*

Human insulin was modified with increasing concentrations of Precursor 1 and loss of biological potency as a function of PEG₅₀₀₀-Fmoc incorporated into insulin was determined.

Insulin (17.24 nmoles in 0.2 ml 0.01 M NaHCO₃) reacted with increasing concentrations of PEG₅₀₀₀-Fmoc-OSu at a molar excess over the protein as indicated in Fig. 4A for 2 hours at 25°C. The degree of derivatization was quantitated by determining the number of free amino groups that remained unmodified and were available for reaction with TNBS. Theoretically, insulin can incorporate 3 moles of PEG₅₀₀₀-Fmoc on the amino side chains of Lys B29, PheB1 and GlyA1. As shown in Fig. 4A, upon reacting insulin with 0.6, 1.3, 1.9, 2.5 and 5.0 molar excess of PEG₅₀₀₀-Fmoc-OSu, 0.3±0.03, 0.59±0.05, 1.1±0.1, 1.5±0.2 and 2.2±0.2 moles of PEG_{5,000}-Fmoc were incorporated into insulin, respectively, indicating that two (of the three) amino side chains of insulin are readily accessible for derivatization.

Aliquots containing 0.4, 0.7, 1.1, 1.5 and 2.2 moles PEG₅₀₀₀-Fmoc covalently attached per mole insulin, were assayed for their biological potencies in a lipogenic assay in rat adipocytes. Under the assay conditions, human insulin stimulates lipogenesis, 4-6 times above basal levels with ED₅₀ value of 0.2±0.02 ng/ml. An insulin derivative exhibiting ED₅₀ of 2.0±0.2 ng/ml in this assay is considered as having 10% the lipogenic potency of native insulin. The results in Fig. 4B show that the respective biological potencies were 87±4, 60±3, 8±1, 4±0.3 and less than 1% when 0.3, 0.6, 1.1, 1.5 and 2.2 mol PEG₅₀₀₀-Fmoc/mol insulin has been incorporated.

20

28(ii). PEG₅₀₀₀-Fmoc-insulins undergo time-dependent reactivation

Insulin conjugates containing one and two moles of PEG₅₀₀₀-Fmoc/mole insulin were incubated for different times at a concentration of 0.172 μM in 0.1 M NaHCO₃ -0.5% bovine serum albumin (BSA) and 1 mM NaN₃ (pH 8.5) at 37°C. The results are shown in Fig. 5. At the indicated time points, aliquots were analyzed (in several concentrations for each aliquot) for their lipogenic potencies in rat adipocytes. With (PEG₅₀₀₀-Fmoc)₂-insulin, no reactivation could be observed in the first 10 hours of incubation. Lipogenic activity then increased, reaching 45±3 and 90% of the native insulin potency at 30±2 and 80±4 hours, respectively.

With (PEG₅₀₀₀-Fmoc)₁-insulin, no such lag period was observed. Activity was regenerated slowly but continuously yielding 16, 24, 30, 36 and 95% of the native potency following 2, 4, 10, 17 and 80 hours of incubation, respectively. Insulin containing about one mole of PEG₅₀₀₀-Fmoc/mol insulin (PEG₅₀₀₀-Fmoc₁-
 5 insulin) was therefore selected for further studies. Mass-spectrum and analytic HPLC analyses revealed that this preparation contains predominantly monomodified derivatives of insulin (MW=11,096 kDa), the remainder being unmodified insulin (about 5%, MW=5,813 kDa) and small quantities of bis (MW=16,587 kDa) and tris (MW=22,661 kDa) modified derivatives.

10 As shown in Fig. 4B, (PEG₅₀₀₀-Fmoc)₁-insulin has 7±1% the biological potency of the native hormone prior to undergoing PEG-Fmoc hydrolysis. Based on these findings *in vitro*, (PEG₅₀₀₀-Fmoc)₁-insulin was administered *in vivo* at ten times higher concentrations than the native hormone, in order to obtain the same glucose-lowering effect (see below).

15

28(iii). (PEG₅₀₀₀-Fmoc)₁-insulin facilitates prolonged glucose-lowering action in vivo

Native insulin (Zn²⁺ free, 1.72 nmol/mouse) or (PEG₅₀₀₀-Fmoc)₁-insulin (17.2 nmoles/mouse) were administered subcutaneously to groups of mice (n=5 in
 20 each group), and the glucose lowering profiles were determined. The results are shown in Fig. 6. Native insulin reduced blood glucose level maximally at 30 min. Circulating glucose levels then returned to normal values with $t_{1/2}$ =1.8±0.2 hours. Following subcutaneous administration of (PEG-Fmoc)₁-insulin, circulating glucose levels were lowered maximally at 4 hours, and were then maintained at the low
 25 glucose level for an additional 4 hours, before returning to the normal values with a $t_{1/2}$ = 12±1 hours (Fig. 6).

28(iv) (PEG₅₀₀₀-Fmoc)₁-insulin manifests prolonged glucose lowering action also after intraperitoneal administration

The prolonged action of subcutaneously administered (PEG₅₀₀₀-Fmoc)₁-insulin can be attributed, theoretically, to a slower absorption rate from the subcutaneous compartment to the circulation as well as to the low fraction of the administered material exposed to receptor-mediated degradation and hydrolysis of the inactive to the active species. In order to differentiate between these factors, the subcutaneous compartment was bypassed by administering native insulin (Zn²⁺-free, 0.345 nmoles/mouse, in 0.2 ml PBS buffer) or (PEG₅₀₀₀-Fmoc)₁-insulin (3.45 nmoles/mouse, in 0.2 ml PBS buffer) intraperitoneally. The glucose lowering capacities were then monitored. Blood glucose levels were determined at different time points.

The results are shown in Fig. 7. Following administration of insulin, circulating glucose levels fell maximally at 15 min and returned to normal level with a $t_{1/2}$ value of 1.0 ± 0.1 hours. In the case of intraperitoneally administered (PEG₅₀₀₀-Fmoc)₁-insulin, circulating glucose level declined more gradually, reaching a maximal fall at 1.0 hour. Levels were then gradually elevated, showing a $t_{1/2}$ value of 3.4 ± 0.1 hours, and returning to normal values only 6 hours after administration (Fig. 7).

Example 29: Biological activity of PEG-FMS-exendin conjugates

Exendin-4 is an insulinotropic glucagon-like peptide-1 (GLP-1) agonist associated with the β -pancreatic cells, elevates endogenous cAMP levels, enhances secretion of insulin, and lowers circulating glucose levels (Eng et al., 1992; Goke et al., 1993; Schepp et al., 1994; Fehmann et al., 1994). A profound pharmacological advantage of this GLP-1 agonist is that, following administration at any dosage, the circulating blood glucose level (BGL) never falls below a threshold glucose level that, in non-diabetic healthy CD1-mice, is 74 ± 7 mg/dl (Shechter et al., 2003).

According to the present invention, the conjugate PEG₄₀₀₀₀-FMS-exendin-4 was prepared (Example 19). Exendin-4 contains one His N α amino function and two Lys N ϵ amino groups, enabling modification at these three positions. Indeed, N-terminal amino acid sequencing revealed that, although the PEG₄₀₀₀₀-FMS-

exendin-4 product eluted as a single peak on HPLC, it was actually a mixture containing primarily the N α -modified hormone. However, in view of the pegylation reaction's reversibility according to the present invention, regenerating the native peptide and protein hormones in physiological environment, this point deserves
5 only minor consideration.

Materials and Methods

(i) *Materials.* Exendin-4 and a non-lysine-containing synthetic irrelevant peptide of 27 amino acids (SEQ ID NO: 14) were synthesized by the solid phase
10 method using a multiple-peptide synthesizer, AMS 422 (Abimed Analyser Technik GmbH, Langenfeld, Germany). An Fmoc-strategy was employed throughout the peptide-chain assembly. 5,5-dithiobis(2-nitrobenzoic acid) (DTNB), reduced glutathione (GSH) and trinitrobenzene sulfonic acid (TNBS) were purchased from Sigma Chemical Co., (St. Louis, MO, USA). All other materials used in this study
15 were of analytical grade.

PEG₄₀₀₀₀-FMS-exendin-4 and PEG₅₀₀₀-FMS-exendin-4 were prepared as described in Examples 18 and 19, respectively.

For the preparation of PEG₄₀₀₀₀-exendin-4, exendin-4 (0.3 mg, 75 nmol) was dissolved in PBS (pH=7.5) and reacted with PEG₄₀₀₀₀-OSu (20 mg, 470 nmol) for 3
20 h, filtered ($\times 7$) through Centricon (cut off =50 kDa) and characterized by MALDI-TOF mass spectrometry (found 48074 Da).

PEG₄₀₀₀₀-FMS-Peptide 27 was prepared as described for PEG₄₀₀₀₀-FMS-exendin-4.

A Centricon-50 ultrafiltration device for aqueous solutions was purchased
25 from Millipore S.A. (France).

Ultraviolet spectra were obtained by Beckman DU 7500 spectrophotometer in 1 cm path length UV cuvettes. Mass spectra were determined using MALDI-TOF and ESMS techniques (Bruker-Reflex-Reflectron model, Germany, and VG-platform-II electrospray single quadrupole mass spectrometer, Micro Mass, U.K.,
30 respectively).

(ii) *HPLC analyses* were performed using a Spectra-Physics SP8800 liquid chromatography system equipped with an Applied Biosystems 757 variable wavelength absorbance detector, and a Spectra-SYSTEM P2000 liquid chromatography system equipped with a Spectra-SYSTEM AS100 auto-sampler and a Spectra-SYSTEM UV1000, all controlled by a ThermoQuest chromatography data system (ThermoQuest Inc., San Jose, CA, USA). The column effluents were monitored by UV absorbance at 220 nm. Analytical RP-HPLC was performed using a pre-packed Chromlith™ Performance RP-18e (4.6 x 100mm, Merck, Darmstadt, Germany). The column was eluted with a binary gradient of 10-100% solution B over 10 min with a flow rate of 3 ml/min (solution A was 0.1% TFA in H₂O and solution B was 0.1% TFA in acetonitrile:H₂O; 3:1, v:v). PEGylated compounds were analyzed using a RP-4 column (250x4 mm, 5 µm bead size, VYDAC, Hesperia, CA, USA) with a binary gradient of 10-100% solution B in 50 min at a flow rate of 1 ml/min.

(iii) *Preparative separations* were performed with pre-packed VYDAC RP-18 or RP-4 columns (250x22 mm; Hesperia, CA). The column was eluted with 10-100% solution B over 60 min (12 ml/min).

(iv) *Glucose-lowering assay*. Three groups of CD1 mice (n=6 per group) were subcutaneously administered with saline, native exendin-4 (4 µg/mouse) or PEG₄₀₀₀₀-FMS-exendin-4 (4 µg peptide/mouse). Circulating glucose levels were measured with a glucose analyzer (Beckman Instrument, Fullerton, CA, USA) by the glucose oxidase method. Blood samples for the blood glucose analyses were taken from the tail veins. The level of glucose in normal healthy CD1-mice was 140±7 mg/dl (7.77 mM).

25

29(i) PEG-FMS-exendin-4 releases exendin-4 upon incubation.

A solution of PEG₄₀-FMS-exendin-4 (0.25 mM, 1ml/ml in terms of exendin-4 in 0.1 M NaHCO₃, pH 8.5) was incubated at 37° C. At different time points, aliquots (50 µl) were withdrawn and analyzed for the release of exendin-4 from the conjugate, using HPLC on a RP-4 column. As shown in Fig. 8, exendin-4 was

30

released from the conjugate [PEG₄₀-FMS]₂-exendin-4 in a slow, homogeneous fashion, with a $t_{1/2}$ of 16 ± 2 h. Exendin-4 was fully released from the conjugate after 48 h of incubation.

5 *29(ii) The hydrolysis rates and reaction orders of PEG₄₀-FMS-exendin-4, PEG₅₀₀₀-FMS-exendin-4 and PEG₅₀₀₀-FMS-4-nitro-phenethylamine*

The hydrolysis rates and reaction orders of the PEG-FMS conjugates PEG₅₀₀₀-FMS-4-nitro-phenethylamine, PEG₅₀₀₀-FMS-exendin-4 and PEG₄₀-FMS-exendin-4, and one MAL-FMS conjugate, N-(MAL-FMS)-Peptide 27, were
10 evaluated at pH = 8.5, 37°C. The structures and half-lives of the conjugates are presented in Table 3. PEG₅₀₀₀-FMS-4-nitro-phenethylamine was prepared as described in Example 20. The conjugate MAL-FMS-Peptide 27 was prepared by reaction of MAL-FMS-NHS with the non-relevant peptide 27 (SEQ ID NO:14).

Fig. 9 shows the hydrolysis of PEG₅₀₀₀-FMS-exendin-4 (circles) and of
15 PEG₅₀₀₀-FMS-4-nitro-phenethylamine (squares) after incubation in PBS at pH 8.5, 37°C. Results in Figs. 8 and 9 are expressed as percent of the maximal peak area of released exendin-4 and 4-nitrophenethylamine, as a function of time.

The hydrolysis rate at pH 8.5 is equivalent to the hydrolysis rate in serum. The release of 4-nitro-phenethylamine and exendin-4 was monitored by RP-HPLC
20 and was determined to be a first order reaction (Table 3 and Figs. 8, 9). As shown (Figs. 8-9, Table 3), the peptides and protein were released from the conjugates in a slow homogenous fashion, with $t_{1/2}$ of 9.4 (PEG₅₀₀₀-FMS-4-nitro-phenethylamine), 13.8 (PEG₅₀₀₀-FMS-exendin-4), and 11.9 (PEG₄₀₀₀₀-FMS-exendin-4). Exendin-4 was fully released from the PEG₄₀ conjugate after 48 h of incubation.

25 The conjugate MAL-FMS-Peptide 27 is TNBS negative as the sole α -amino moiety of Peptide 27 was derivatized. A solution of MAL-FMS-Peptide 27 (0.5 mM in 0.1 M NaHCO₃, pH 8.5) was incubated at 37°C. Aliquots (0.2 ml) were withdrawn at different time points and analyzed for the appearance of the free α -amino group using TNBS. The FMS-MAL (N-ethyl maleimide) moiety was
30 hydrolyzed in a slow and nearly homogeneous fashion from the α -amino moiety of

the peptide, with a $t_{1/2}$ value of 8.4 h. Hydrolysis was complete after 32 hrs of incubation.

5 *29(iii) PEG₄₀-FMS-exendin-4 facilitates prolonged glucose lowering action in mice.*

Figs. 10A-10B show the glucose-lowering profile of subcutaneously administered native exendin-4 and of PEG₄₀-FMS-exendin-4, both at a dose of 10 μg (Fig. 10A) or 4 μg (Fig. 10B) peptide/mouse relative to a saline-treated group of mice.

10 Fig. 10A shows the glucose lowering profile of subcutaneously administered native-exendin-4 and of PEG₄₀-FMS-exendin-4, both at a dose of 10 $\mu\text{g}/\text{mouse}$. With the native peptide, blood glucose levels declined from 139 ± 10 to 96 ± 7 within 0.5 h reaching a maximal fall in 2-4 hours after administration (74 mg/dl). The return to initial glucose level was then proceeded with a $t_{1/2}$ value of 14.2 ± 1 h.

15 Following the subcutaneous administration of PEG₄₀-FMS-exendin-4, little decrease in blood glucose level is seen at 0.5 h after administration. Circulating glucose level then fall gradually, with the lowest glucose concentration reached at 6-8 hours after administration (80-90 mg/dl). Stable, low circulating glucose concentrations then maintained over nearly 50 hours, prior to the return to initial

20 glucose levels with a $t_{1/2}$ value of 70 ± 3 hrs.

Fig. 10B shows the glucose lowering profile of subcutaneously administered native-exendin-4 and of PEG₄₀-FMS-exendin-4, both at a dose of 4 $\mu\text{g}/\text{mouse}$. With the native peptide, blood glucose levels declined by 26-28% (from 140 mg/dl to 104-101 mg/dl), with the largest percent change in blood glucose levels occurring

25 0.5-1 h after administration. Glucose concentrations then returned to their initial levels with a $t_{1/2}$ value of 3.7 ± 0.3 h. Following the subcutaneous administration of PEG₄₀-FMS-exendin-4, the decrease in blood glucose level took place at a more moderate rate. Circulating glucose reached its lowest concentration 8-12 hours after administration (92 mg/dl, 33%). Stable, low circulating glucose concentrations were

30 then maintained for a further 12 hours. Return to initial glucose levels took place

with a $t_{1/2}$ of 30 ± 2 h, being 7.5 times longer than that obtained by the same dose of the native hormone.

Calculations based on the hydrolysis rates of FMS-exendin-4 (described in US Patent Application No. 10/408,262) and PEG₄₀₀₀₀-FMS-exendin-4 at 37°C in normal sera and PBS (pH 8.5), respectively, revealed that ~ 4% of exendin-4 is released from the conjugate each hour *in vivo* (Shechter et al., 2001). We further hypothesized that this release rate, if combined with prolonged maintenance of the conjugate in the circulatory system, prior to exendin-4 hydrolysis, should yield a long-lasting glucose-lowering signal in mice, as indeed is found following a single subcutaneous administration of PEG₄₀₀₀₀-FMS-exendin-4 conjugate (Fig. 10B). Irreversibly conjugated PEG₄₀₀₀₀-exendin-4 has only $\leq 1\%$ of the activity of native exendin-4 ($IC_{50} = 250 \pm 30$ pmol/mouse versus $IC_{50} = 2.5 \pm 0.24$ pmol/mouse for the native exendin-4, not shown).

15 **Example 30. Biological activity of PEG₄₀-FMS-IFN α 2**

Type I interferons (IFNs) are proteins that initiate antiviral and antiproliferative responses. Interferons are clinically important, and several subtypes of IFN α 2 were approved as drugs for the treatment of hepatitis B and C as well as for cancers such as chronic myelogenous leukemia and hairy cell leukemia. Interferons regulate signals through the Janus tyrosine kinase (Jak/STAT proteins), and by reducing phosphorylation and activation of MEK1 and ERK1/2 through a *Ras/Raf* independent pathways (Romerio et al., 2000). Human type I interferons induce differential cellular effects, but act through a common cell surface receptor complex comprised of the two subunits, Ifnar1 and Ifnar2. Human Ifnar2 binds all type I IFNs, but with a lower affinity and specificity than the Ifnar complex. Human Ifnar1 has a low intrinsic binding affinity towards human IFNs, but modulates specificity and affinity of other ligands of the Ifnar complex (Cutrone and Langer, 2001).

IFN α 2 may be administered intramuscularly, subcutaneously or intravenously, resulting in different pharmacokinetic profiles. In any mode, the

administered cytokine is rapidly inactivated by body fluids and tissues (O'Kelly et al., 1985), and cleared from the blood plasma several hours following administration (Rostaing et al., 1998). The major routes of IFN α 2 elimination from the circulatory system are through proteolysis, receptor mediated endocytosis and kidney filtration
5 (Goodman and Gilman, 2001).

Prolonging the maintenance dose of IFN α 2 in circulation is a desirable clinical task. A non-reversible, 12 kDa-PEG-IFN α 2 conjugate, has been therapeutically approved in 2001. It is administered once a week to hepatitis C patients, and facilitates a sustained anti-viral response rate of 24%, as opposed to a
10 12% response rate obtained by the native cytokine (Schering-Plough Corporation, 2001 press release; Baker, 2003). However, while the covalent attachment of PEG chains to proteins prolongs their lifetime *in vivo*, it often results in a dramatic reduction or even loss of biological and pharmacological activities (Fuertges and Abuchowski, 1990; Katre, 1993; Bailon and Berthold, 1998; Nucci et al., 1991;
15 Delgado et al., 1992; Fung et al., 1997; Reddy, 2000; Veronese, 2001). The pegylated formulation of IFN α 2 currently in use has 7% the activity of the native cytokine, calling for higher doses to be administered (Bailon et al., 2001). Furthermore, PEG-IFN does not readily penetrate all tissues: while 12 kDa PEG-IFN α 2b is widely distributed, 40 kDa -PEG-IFN α 2a is restricted to the blood and
20 the interstitial fluid (Glue et al., 2000; Reddy et al., 2002). This major drawback can be overcome by designing a PEG-IFN α 2 conjugate capable of generating native IFN α 2 at a slow rate under physiological conditions.

These problems of the prior art can be overcome by the mono- and bis-PEG₄₀-FMS-IFN- α 2 conjugates of the invention, in which IFN- α 2 is linked to the
25 PEG moiety through the FMS moiety that provides the slowly hydrolysable bond. These novel reversibly pegylated-conjugates and their prolonged anti-viral activity *in vivo* are discussed here in detail.

Materials and Methods

(i) *Materials.* Non-glycosylated human IFN- α 2 was prepared as described in WO 02/36067 as previously described by Piehler and Schreiber (1999). Preparation of PEG₄₀-FMS-IFN α 2 and (PEG₄₀-FMS)₂-IFN α 2 is described in Examples 21 and 22, respectively.

(ii) *Receptor binding affinities* were evaluated by BIAcore (SPR Detection) measurements. The BIAcore[®] 3000 system, sensor chips CM5, HBS (10 mM HEPES, 3.4 mM EDTA, 150 mM NaCl, 0.05% surfactant P20, pH 7.4) and the amine coupling kit were from BIAcore (Sweden). Chip immobilization by Ifnar2 and the BIAcore measurements were carried out according to Piehler and Schreiber (2001). In short, Ifnar2-EC was immobilized to the surface using the non-neutralizing anti-Ifnar2-EC (Ifnar2-extra cellular) mAb 46.10, followed by cross-linking with a second mAb (117.7) (kindly provided by D. Novick, Weizmann Institute of Science, Rehovot, Israel). The binding curves were evaluated with the BIAevaluation software (Biacore AB, Sweden) using a simple one-to-one kinetic model. Increase in RU (resonance unit) after specific binding to the receptor corresponds to the amount of protein bound to the sensor surface. To estimate the increase in RU resulting from the nonspecific effect of the protein on the bulk refractive index, binding of the protein to a control surface with no immobilized ligand was also measured and subtracted. For the determination of the active interferon concentration the equilibrium response was plotted against the estimated initial concentration. The data were fitted using KaleidaGraph (version 3.0.4, Abelbeck Software) using the equation:

$$y = \frac{1 \cdot 10^8 * m1 * m2}{1 \cdot 10^8 * m1 * m0 + 1}$$

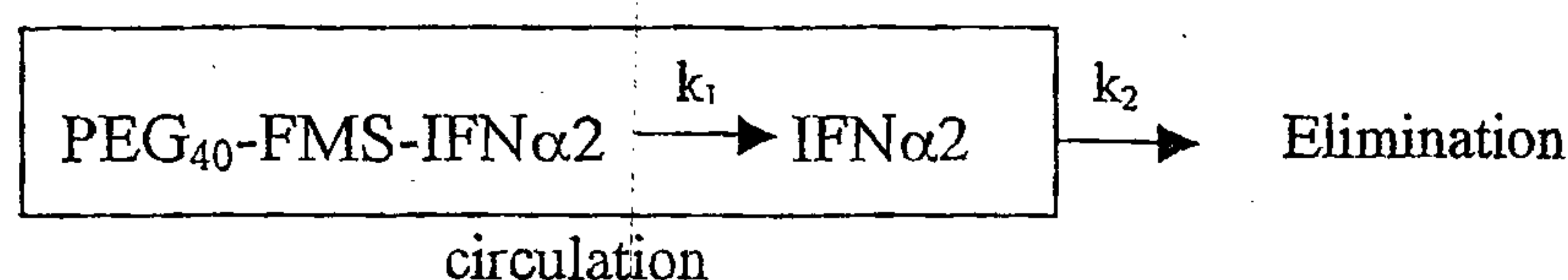
whereby a K_A of 1×10^8 was determined independently for IFN α 2 binding and fixed for all samples, $m1$ is Ru/Rmax (the percent of active interferon measured in the sample), $m2$ is Rmax, and $m0$ is the observed Ru.

(iii) *In vivo experiments* were performed using male Wistar rats (150-170 g). Rats were injected either subcutaneously or intravenously (0.2 ml/rat).

(iv) *Antiviral activity of IFN α 2 and its derivatives* was determined by the capacity of the cytokine to protect human amnion WISH cells against Vesicular Stomatitis Virus (VSV)-induced cytopathic effects (Rubinstein et al., 1981).

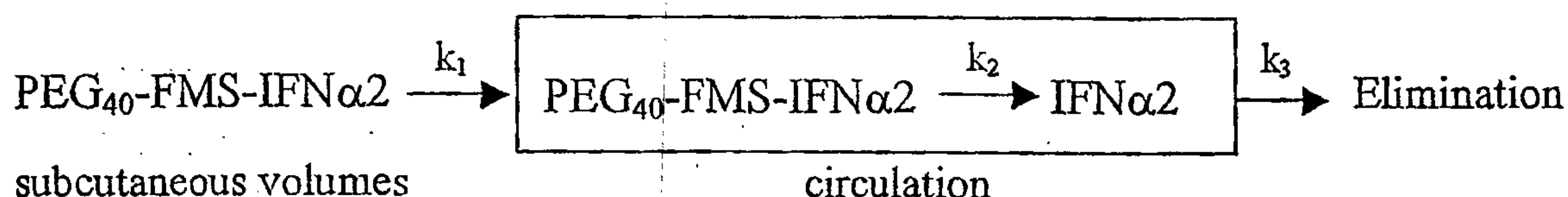
(v) *Simulations of experimental data* were performed using Pro-Kineticist II, a 2nd Order Global Kinetic Analysis software (Applied Photophysics Ltd., England).

For the *i.v.* administration, the following model was considered:



Whereby, $k_1=0.01 \text{ hr}^{-1}$ and $k_2=0.65 \text{ hr}^{-1}$ (determined experimentally by antiviral assay).

10 For the *s.c.* administration, the following model was considered:



Whereby, $k_1=0.02 \text{ hr}^{-1}$, $k_2=0.01 \text{ hr}^{-1}$, and $k_3=0.65 \text{ hr}^{-1}$.

k_1 , which could not be determined directly, was estimated to be 0.02 hr^{-1} from the fit of the simulation to the experimental data.

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30(i) *PEG₄₀-FMS-IFN α 2 has modified receptor-binding capacity*

The binding capacity of the modified IFN α 2 of the invention toward the immobilized recombinant extracellular part of IFN α 2 receptor (ifnar2-EC) was monitored under flow-through conditions by an optical probe called reflectometric interference spectroscopy (RIFS). This method detects biomolecular interactions of ligands to transducer-bound proteins as a shift in the interference spectrum caused by change of the apparent optical thickness of the transducer chip. A shift of 1 pm corresponds to approximately 1 pg/mm^2 protein on the surface. The transducer surface was modified with a dextran layer and carboxylated by reaction with molten

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glutaric anhydride (Sigma) at 75° for 2-8 h. On such surfaces, electrostatic pre-concentration and covalent immobilization of proteins were carried out by standard BIAcore protocols. After this procedure, ifnar2-EC was immobilized into a carboxylated dextran layer. All measurements were carried out in 50 mM Hepes (pH 7.4) containing 150 mM NaCl and 0.01% Triton X-100. A sample of 0.8 ml was injected for 80 s with a data acquisition rate of 1 Hz. Flow rates of 50 µl/s were applied. Under these conditions, the samples in the flow cell were exchanged within one second, allowing the analysis of processes within 5 seconds.

The results are summarized in Table 4. Monomodified and bis-modified conjugates of PEG₄₀-FMS-IFNα₂ had 9±1 and 0.4±0.05% the receptor-binding capacity of IFNα₂, respectively. These conjugated derivatives, however, underwent time-dependent reactivation upon incubation at 37°C in phosphate-buffer pH 8.5 or in normal human serum (not shown). Reactivation proceeded with a t_{1/2} value of 9±1 and 24±3 hrs for the mono and bis-pegylated derivatives, respectively reaching nearly full reactivation following 50 hours of incubation (Table 4).

30(ii) PEG₄₀-FMS-IFNα₂ releases native-active IFNα₂ upon incubation, at a rate constant of 0.01hr⁻¹.

In the set of experiments summarized in Figs. 11A-C, PEG₄₀-FMS-IFNα₂ was incubated in 0.1 M phosphate buffer in the presence or absence of 0.6% BSA and 2 mM sodium azide (NaN₃) (pH 8.5, 37 °C). At this pH value, the rate of FMS hydrolysis from FMS-proteins is nearly identical to that obtained in normal human serum, *in vitro*, or in the circulatory system *in vivo* (Gershonov et al., 2000; Shechter et al., 2001 and 2002). Aliquots were drawn at different time points and analyzed for the release of IFNα₂ from the conjugate by SDS-PAGE (Fig. 11A) and by BIAcore, measuring the active concentration of Ifnar2 according to the law of mass action (Fig. 11B). The interferon-binding curve on the Ifnar2 surface resembles that of a homogeneous population of native interferon, suggesting that PEG₄₀-FMS-IFNα₂ does not bind Ifnar2. For the SDS-PAGE analysis, the amounts

of IFN α 2 discharged were quantified relative to an IFN α 2 reference of known concentration and intensity. In both cases the discharge profiles are in good agreement. The 10% active interferon observed at time zero in the BIAcore profile is due to native interferon present in the sample. The rate of discharge was determined by fitting the quantity of active interferon to a single exponential equation (Fig. 11C). Accordingly, IFN α 2 is released from the conjugate with a rate constant of 0.01hr⁻¹ (Fig. 11). Upon 66 hours of incubation, 50% of the IFN α 2 in the conjugate is discharged and is fully active. From the extrapolation of the curve fit obtained, it is assumed that nearly all of the interferon will eventually be released and regain full activity. No BIAcore data was collected at very long time points as keeping proteins for weeks at 37 °C is not advisable.

30(iii) Subcutaneous administration of PEG₄₀-FMS-IFN α 2 dramatically increased its half-life in vivo.

Next, we determined the half-life and activity of PEG₄₀-FMS-IFN α 2 in rats. Human IFN α 2 is not active in rats, albeit its concentration can be determined from the antiviral potency in rat serum by measuring the VSV-induced cytopathic effects in WISH cells (see Methods above, antiviral activity assay). Native IFN α 2 or PEG₄₀-FMS-IFN α 2 were administered subcutaneously to rats. Blood aliquots were drawn at various time points, and analyzed for their antiviral potency. Following the administration of the native unmodified IFN α 2 (100 μ g/rat), circulating antiviral activity declined with a $t_{1/2}$ value of ~1 hr reaching a level lower than 20 pM IFN α 2, 12 h after administration (Fig. 12).

The circulatory behavior of PEG₄₀-FMS-IFN α 2 following subcutaneous administration to rats (at doses of 12 μ g/rat, 60 μ g/rat, or 120 μ g/rat) shows a clearly visible dose-dependent behavior (Fig. 12). Administration of 12 μ g/rat of the conjugated IFN α 2 yielded maintenance levels of 70 \pm 10 pM IFN α 2 which were maintained 56 hours following administration. When a 10-fold increase in PEG₄₀-FMS-IFN α 2 was administered, IFN α 2 was continuously present in the serum 56

hours at a molar concentration of 450 pM. Administration of 60 $\mu\text{g}/\text{rat}$ of the conjugate resulted in interferon levels of 225 pM at 56 hours, and 25 pM at 200 hours. Native IFN α 2 present in the administered sample (approximately 10% as determined by BIAcore) contributed to the initially high levels of IFN α 2 observed in the rats' serum. These values display a clearance curve similar to that of native IFN α 2. The remaining 90% of the IFN α 2 were slowly discharged from the conjugate.

30(iv) Intravenous administration of PEG₄₀-FMS-IFN α 2 to rats.

To eliminate the contribution of the subcutaneous exchange, both the conjugate and the native cytokine were administered to rats (30 $\mu\text{g}/\text{rat}$) intravenously. For native interferon, the same half-life was measured, indicating that it readily penetrates the circulatory system following subcutaneous administration (Fig. 13). For PEG₄₀-FMS-IFN α 2, antiviral activity was still detected 150 hours following intravenous administration, demonstrating the prolonged effects of the pegylated cytokine. Discharged IFN α 2 level of 10 pM still remained 150 hours post administration, while native IFN α 2 was eliminated within 30 hours post-administration. It should be noted that the large shoulder observed following subcutaneous administration of the conjugate (Fig. 12) is not observed when PEG₄₀-FMS-IFN α 2 was administered intravenously (Fig. 13).

30(v) Simulations of experimental data.

Using the rate constants obtained from both BIAcore data ($k = 0.01 \text{ hr}^{-1}$ for the discharge of IFN α 2 from the PEG conjugate) and the antiviral activity assay of native IFN α 2 ($k = 0.65 \text{ hr}^{-1}$ for the elimination of interferon), both the subcutaneous and intravenous administration modes of PEG₄₀-FMS-IFN α 2 to rats were simulated (Figs. 14A and 14B, respectively). In both cases, the simulated data and the experimental results were in good agreement. As became evident from the simulated data, the passage of the conjugate, but not of the native interferon, from

the subcutaneous volume to the bloodstream, proceeds at a slow rate and is in the order of the discharge of interferon from the conjugate. This explains the shoulder observed between 10-70 hours in the active protein concentration. As expected, this shoulder is not found when PEG₄₀-FMS-IFN α 2 is administered intravenously.

5

Discussion

As mentioned before, the pegylating technology applied to therapeutic proteins often leads to a drastic loss in the biological and the pharmacological potencies of the conjugates. In principle, this deficiency can be overcome by
10 introducing the PEG chains via a chemical bond that is either sensitive to hydrolysis or can be cleaved enzymatically by serum proteases or esterases. A prerequisite condition for efficient pegylation is that the hydrolysis of the PEG chains from the conjugate is to take place at a slow rate, and in a homogenous fashion *in vivo*.

Two basic irreversible PEG-IFN α 2 conjugates are in therapeutic use at
15 present. The first, a 12 kDa-PEG-IFN α 2, which satisfactorily permeates into tissues. This preparation, however, is a relatively short-lived *in vivo*, since its low molecular mass (calculated mass = 32 kDa) is insufficient to markedly arrest kidney filtration. The second formulation, a 40 kDa-PEG-IFN α 2, is an extremely long-lived species *in vivo*. This conjugate, however, has poor permeability into tissues.
20 Following administration, the conjugate is distributed only in the blood and in the intestinal fluids (Bailon et al., 2001). We have, therefore, anticipated that the two prerequisite features for an optimal PEG-IFN α 2 conjugate, namely, a prolonged maintenance *in vivo* combined with free access to peripheral tissues, can be obtained by linking a slowly hydrolyzable PEG₄₀-chain to IFN α 2.

25 We have previously found that upon linkage of FMS to proteins, the FMS-protein conjugate undergoes hydrolysis at physiological conditions with a desirable pharmacokinetic pattern (Gershonov et al., 2000; Shechter et al., 2001, 2002, 2003). The rate of FMS hydrolysis is dictated exclusively by the pH and the nucleophilicity of the serum, both of which are maintained in mammals under strict
30 homeostasis (Shechter et al., 2001). We therefore based our new development on

the FMS principle. In neutral, aqueous solutions, FMS moieties undergo slow, spontaneous hydrolysis, resulting in the regeneration of the native proteins (Shechter et al., 2001). For this purpose, NHS-FMS-MAL was synthesized, enabling us to link sulfhydryl-containing PEG chains to the amino groups of peptides and proteins via the hydrolyzable FMS function. The working hypothesis was that an inactive PEG-interferon conjugate could regenerate the native protein in its active form in a continuous fashion over a long period of time. The principal monomodified PEG₄₀-FMS-IFN α 2 conjugate obtained (Table 2) is devoid of the cytokine binding potency and can therefore be referred to as a 'prodrug'. Upon incubation, the native cytokine is released by hydrolysis, and the binding potency of IFN α 2 to Ifnar2 is regenerated with a rate constant of 0.01hr⁻¹.

A single subcutaneous administration of PEG₄₀-FMS-IFN α 2 significantly prolonged the levels of IFN α 2 in the serum of rats. While IFN α 2 was short-lived *in vivo*, having a half-life of ~1 hr, the PEG₄₀-FMS-IFN α 2 conjugate exerted its antiviral activity over a period of 200 hours. Furthermore, there is a dose-dependent ratio between the quantity administered and the active interferon levels over a prolonged duration *in vivo*. This observation is beneficial for optimization of dosing regimens in future clinical use.

It should be noted that the IFN α 2 molecule contains 13 amino functions theoretically available for PEG₄₀-FMS attachment. The exact site of pegylation was not determined. In view however, of its reversibility and the generation of the native protein, it seems that this point deserves a rather minor consideration.

In summary, following the new conceptual approach for reversible-pegylation of the invention, whereby a pharmacologically 'silent' conjugate is 'trapped' in the circulatory system and releases the parent protein with a desirable pharmacokinetic profile, we have succeeded in combining prolonged maintenance of IFN α 2 *in vivo* with the release of active-native IFN α 2 to ensure access to peripheral tissues.

30 Example 31: Biological activity of the PEG₄₀-FMS-PYY₃₋₃₆ conjugate

The hypothalamic family of neuropeptide Y (NPY) receptors plays a major role in regulating satiety and food intake (Schwartz, 2000). The putative inhibitory Y2 pre-synaptic receptor (Y2R) is expressed in the arcuate nucleus, which is accessible to local and peripheral agonists of the NPY family (Broberger et al., 5 1997; Kalra et al., 1999). One such Y2R agonist is peptide YY₃₋₃₆ (PYY₃₋₃₆), which is released from the gastrointestinal tract post-prandially in proportion to the caloric content of a meal (Pedersen-Bjergaard et al., 1996; Adrian et al., 1985; Grandt et al., 1994). Recently, it was demonstrated that peripheral administration of PYY₃₋₃₆ inhibits food intake in humans, mice and rats and reduces weight gain in rats 10 [Pittner et al., 2002; Batterham et al., 2002, 2003). Thus, infusion of PYY₃₋₃₆ to reach the normal post-prandial circulatory concentrations of this peptide lead to a peak in serum PYY₃₋₃₆ within 15 min, followed by a rapid decline to normal levels within 30 min. Despite this rapid clearance, administration of PYY₃₋₃₆ to fasting individuals decreases their appetite and reduces food intake by 33% within a 12 h 15 period following PYY₃₋₃₆ administration.. Furthermore, no compensatory food intake occurs over the next 12 h (Batterham et al., 2002). Therefore, PYY₃₋₃₆ may find a clinical use in treatment of obesity and its associated disorders, including type II diabetes mellitus and cardiovascular diseases (Schwartz and Morton, 2002).

The short circulatory half-life of PYY₃₋₃₆ and its potential in management of 20 obesity prompted us to develop a longer-acting form of PYY₃₋₃₆. Indeed, in our hands the satiety induced by PYY₃₋₃₆ in mice lasted for only 2-4 h following subcutaneous (sc) injection. As mentioned before, covalent attachment of PEG to proteins and peptides prolong their half-life *in vivo* but often leads to a drastic loss of their biological or pharmacological activity. We found that PYY₃₋₃₆, pegylated 25 using standard chemistry, i.e. through formation of a stable bond, indeed lost its biological activity. We have then prepared and test here the biological activity of the PEG₄₀-FMS- PYY₃₋₃₆ pro-drug of the invention.

30 Materials and methods

(i) *Animals.* C57BL/6J male mice (Harlan Labs) aged 9 ± 1 week (20-30 g body weight) were used. Mice were kept under controlled temperature (21-23°C) and light conditions (light on 6:00-18:00) at the Weizmann Institute of Science (Rehovot, Israel) animal facility. The mice were acclimated for at least one week prior to the initiation of the study. Mice had free access to drinking water at all times during the experiments. All experimental protocols were in accordance with the Israeli regulations of laboratory animal welfare and were approved by the Institutional Internal Committee for Animal Welfare.

(ii) *Reagents.* Peptide YY₃₋₃₆ was synthesized by the solid phase method, using a multiple-peptide synthesizer AMS 422 (Abimed Analyser Technik GmbH, Langenfeld, Germany). The resulting peptide was HPLC-purified and characterized by MALDI-TOF mass spectroscopy (MS) and N-terminal protein microsequencing. All other reagents were of analytical grade and were purchased from Sigma Chemical Co. (Ness Ziona, Israel).

(iii) *Analytical Procedures.* Mass spectra were determined using MALDI-TOF and Electro Spray (ES-MS) techniques (Bruker-Reflex-Reflectron model, Germany, and VG-platform-II electrospray single quadropole mass spectrometer, Micro Mass, U.K., respectively). PYY₃₋₃₆, PEG₄₀-FMS-PYY₃₋₃₆ and PEG₄₀ were resolved by analytical HPLC (Lichrosorb RP-4 column, 4x250 mm, Merck). Buffer A was 0.1% aq. trifluoroacetic acid (TFA) and Buffer B was 0.1% TFA in 75% aq. Acetonitrile. A gradient of 10-100% Buffer B was used over 50 min. at a flow rate of 1 ml/min. The retention times (Rt) of PYY₃₋₃₆, PEG₄₀-FMS-PYY₃₋₃₆ and PEG₄₀-9-sulfo-fulvene were 21.53, 39.03 and 44.387 min., respectively. Amino acid analyses were performed following 6N HCl hydrolysis at 110°C for 24 h using a Dionex Automatic amino acid analyzer HP1090 (Palo Alto, CA, USA). N-terminal sequence analyses were performed with a Model 491A Procise Protein sequencer (Applied Biosystems, Foster City, CA, USA).

(iv) *Synthetic procedures:*

PEG₄₀-FMS-PYY₃₋₃₆ was prepared as in Example 23.

PEG₄₀-FMS-Glycine ethyl ester (PEG₄₀-FMS-GEE). To a stirred solution of PEG₄₀-SH (0.25 mM in 0.1M phosphate buffer pH 7.2-10 mM Na ascorbate), 292 μ g MAL-FMS-NHS was added (two-fold molar excess over PEG₄₀-SH). After 7 min, 0.2 ml from a solution of 0.5M glycine ethyl ester was added. The reaction was carried out for 1 h, and the mixture was then dialyzed overnight against water. The resulting PEG₄₀-FMS-glycine ethyl ester was characterized and quantitated by its absorbance at 280 nm/ ϵ_{280} = 21,200) and by the amount of glycine in the preparation following acid hydrolysis of a 20 μ l aliquot and amino acid analysis.

PEG₄₀-PYY₃₋₃₆. Irreversibly pegylated PYY₃₋₃₆ was prepared by reacting PYY₃₋₃₆ (1 mg/ml in 0.1 M phosphate buffer pH 7.2) with four equivalents of solid PEG₄₀-OSu, (43 mg). The reaction was carried out for 1 h and the mixture was then dialyzed overnight against water. The conjugate thus obtained contains one PEG₄₀ residue per PYY₃₋₃₆, as determined by MALDI-TOF MS. (PEG₄₀-OSu, 43,626 D; PYY₃₋₃₆, 4,047 D; conjugate, 47,712 D).

(v) *Food intake measurements*: Groups of 10 mice were deprived of food for 24 h and then given excess pre-weighed standard chow for a period of 2 h. Drinking water was provided at all times. The mice received sc injections of either saline (0.1 ml/mouse), native PYY₃₋₃₆ or PYY₃₋₃₆ derivatives at the indicated doses and times within 24 h prior to the start of the 2 h re-feeding period. A minimum interval of 1 h was introduced between injection and re-feeding to avoid a stress-dependent decrease in food intake. The amount of food consumed per group was determined at the end of the feeding period. Remaining chow was weighed after the 2 h re-feeding period and the cumulative food intake per 10 mice was calculated. Values of food intake in replicate experiments were normalized according to the saline controls. Results are expressed as food intake per 10 mice \pm SD from 2-5 replicate experiments.

(vi) *Statistical analysis*. The significance of differences in food consumption was determined by the Student's t-test, using the total weight of food consumed by each group of 10 mice as a single value. A saline-injected group of 10 mice was

included in each experiment as a control. A two-tailed, paired t-test was performed between the control and treated groups.

31(i) The effect of PYY₃₋₃₆ on food intake in mice.

5 To evaluate the duration and magnitude of the effect of PYY₃₋₃₆ and its derivatives on food intake, we employed the mouse re-feeding model of 24 h starvation followed by a re-feeding period of 2 h. Initially we repeated previous studies, where PYY₃₋₃₆ was administered immediately prior to the re-feeding period. However, we found that the stress of mere handling the mice had a profound and
10 inconsistent effect on food intake, thereby reducing the difference between saline and PYY₃₋₃₆. We then injected the mice sc 1 h prior to start of the re-feeding period and obtained a much more consistent difference between PYY₃₋₃₆ and saline. Fig. 15 shows that PYY₃₋₃₆ inhibited food intake in a dose-dependent manner. Mice receiving saline or PYY₃₋₃₆ at a dose of 5 nmol/mouse consumed 10.7±1.26 and
15 5.26±1.47 g chow per 10 mice, respectively ($P<0.001$, $N=5$). These figures correspond to a 50% decrease in food intake by the PYY₃₋₃₆ groups, as compared with the control saline groups. Inhibition was statistically significant at all doses used, including the lowest dose of 0.2 nmol/mouse (0.8 µg/mouse, $P>0.05$, $N=3$). The dose-response corresponded to a half maximal effect of PYY₃₋₃₆ at about 0.5
20 nmol/mouse (Fig. 15).

We then determined the duration of the satiety induced by sc administration of PYY₃₋₃₆ (5 nmol/mouse) at different times prior to the re-feeding period. The satiety induced by PYY₃₋₃₆ was maximal when given 1 h prior to re-feeding and rapidly decreased when PYY₃₋₃₆ was administered at earlier times. The half-life of
25 its biological response was about 3 h and no effect was seen with 5 nmol PYY₃₋₃₆ when administered nearly 10 h prior to the re-feeding period (Fig. 16).

Intraperitoneal (rather than subcutaneous) administration of PYY₃₋₃₆ to mice has induced a considerably shorter-lived satiety effect. For example, an ip dose of 5 nmol PYY₃₋₃₆/mouse was fully effective when administered 30 min before re-
30 feeding, but had no effect when administered 2 h before meal (not shown).

We then attempted to extend the biological activity of PYY₃₋₃₆ by conventional pegylation. Thus, mono-pegylated PYY₃₋₃₆ was prepared (see Materials and Methods above) and tested for its biological activity by sc administration (5 nmol/mouse) 1 h prior to the re-feeding period. No significant reduction in food intake was obtained as compared with the saline control, indicating that conventional pegylation, which results in a stable pegylated peptide, abolished the biological activity of PYY₃₋₃₆ (Fig. 17).

We then tested if the inactivation of PYY₃₋₃₆ was due to the bulkiness of the PEG group or due to blocking of the amino residues of PYY₃₋₃₆. Acetylation of the two amino groups of PYY₃₋₃₆ largely abolished the effect of PYY₃₋₃₆ in inducing satiety *in vivo*. Thus, mice injected with 20 µg Nα-Nε-diacetyl-PYY₃₋₃₆ per mouse ate 10.54 ± 0.08 g chow per 10 mice, as compared with 10.6 ± 1.26 g in the saline group and 5.26 ± 1.47 g in the PYY₃₋₃₆ group. Hence, even attachment of a small acetyl group was sufficient to disrupt the biological activity of PYY₃₋₃₆.

31(ii) Preparation and characterization of PEG₄₀-FMS-PYY₃₋₃₆

The lack of biological activity following pegylation or even acetylation of PYY₃₋₃₆ prompted us to prepare the PEG₄₀-FMS-PYY₃₋₃₆ conjugate described in Example 23 hereinabove. This product was analyzed by mass-spectrometry to determine the ratio of PEG₄₀ to PYY₃₋₃₆ by MALDI-TOF MS analyses. PYY₃₋₃₆ exhibited a molecular mass of 4047.51 D (calc. = 4049.6 D). PEG₄₀-SH had an average mass of 43,626 D, and the conjugate yielded a major peak corresponding to a molecular mass of 47,712.6 D (not shown). The calculated mass of the 1:1 conjugate of PEG₄₀-SH and MAL-FMS-PYY₃₋₃₆ is 48,087 D. Thus the main product corresponds to such a 1:1 conjugate.

31(iii) PEG₄₀-FMS is linked to the α amino group of PYY₃₋₃₆

Peptide YY₃₋₃₆ contains the N-terminal α-amino group of isoleucine and one ε-amino group of lysine at position 2, both of which are potentially available for acylation by MAL-FMS-OSu. To determine the site(s) of acylation, PEG₄₀-FMS-

PYY₃₋₃₆ was reacted with a 500 molar excess of acetic anhydride at pH 7.0 for 1 h, followed by dialysis against water overnight. The PEG₄₀-FMS group was then removed by incubating the acetylated PEG₄₀-FMS-PYY₃₋₃₆ for 4 days at pH 8.5. The product was then subjected to N-terminal protein sequence analysis. As shown in Fig. 18, cycles 1, 2 and 3 yielded the expected amounts of isoleucine, ε-acetyl lysine and proline, respectively. No free lysine was found in cycle 2. Thus, the conjugate consists of PEG₄₀-FMS and PYY₃₋₃₆ at a 1:1 ratio. Furthermore, based on the sequencing yields, the PEG₄₀-FMS group is primarily linked to the N-terminal α-amino group of PYY₃₋₃₆. Nevertheless, this analysis could not entirely exclude the presence of a PYY₃₋₃₆ mono-substituted at its ε-lysine side chain. However, in view of the complete regeneration of the native peptide, this point is of a rather minor significance.

31(iv) PEG₄₀-FMS-PYY₃₋₃₆ hydrolyses to yield PYY₃₋₃₆ under physiological conditions.

To evaluate the rate of PYY₃₋₃₆ release from PEG₄₀-FMS-PYY₃₋₃₆, we incubated the conjugate in phosphate buffer (pH 8.5, 0.1 M, 37°C). Aliquots were withdrawn at different times and subjected to analytical HPLC, using eluting conditions, which resolve PEG₄₀-FMS-PYY₃₋₃₆ from PYY₃₋₃₆. At pH 8.5, the rate of FMS hydrolysis from FMS-peptides or proteins is nearly identical to that obtained in normal human serum (Shechter et al., 2001). As shown in Fig. 19, PYY₃₋₃₆ was released from the conjugate in a slow, nearly homogenous fashion with a half-life of 5.3 h. After 40 h, the cumulative amount of free PYY₃₋₃₆ reached 79% of the input PEG₄₀-FMS-PYY₃₋₃₆.

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31(v) Hydrolysis of PEG₄₀-FMS-PYY₃₋₃₆ in normal mouse serum.

We found that PYY₃₋₃₆ undergoes rapid proteolysis in normal mouse serum at 37°C with a half-life of 3±0.7 min. (Fig. 20, insert). Therefore, the rate of PEG₄₀-FMS-PYY₃₋₃₆ hydrolysis was evaluated by the amount of 2-PEG₄₀-9-sulfo-fluorene released during hydrolysis. PEG₄₀-FMS-PYY₃₋₃₆ was incubated in normal mouse

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serum at 37°C. Aliquots were withdrawn at different times, ethanol (3 volumes) was added to precipitate serum proteins, and following centrifugation the amount of 2-PEG₄₀-9-sulfo-fluorene was measured in the supernatants by HPLC. As shown in Fig. 20, hydrolysis of PEG₄₀-FMS-PYY₃₋₃₆ in normal mouse serum at 37°C proceeded in a nearly homogenous fashion with a half-life of 7.0±0.3 h.

31(vi) PEG₄₀-FMS-PYY₃₋₃₆ induces a prolonged satiety in mice.

We then determined the duration of the effects of PEG₄₀-FMS-PYY₃₋₃₆ (5 nmol/mouse) on food intake. The half-life of the biological response of PYY₃₋₃₆ was about 3 h (Fig. 16). By comparison, the biological activity of PEG₄₀-FMS-PYY₃₋₃₆ was much more persistent (Fig. 21). Mice given PEG₄₀-FMS-PYY₃₋₃₆ 18 h prior to re-feeding ate 4.7±0.14 g chow per 10 mice, a value representing a 52% reduction in food intake as compared with the control (saline) group ($P>0.05$). A statistically significant reduction of 27% in food intake was also seen in mice given PEG₄₀-FMS-PYY₃₋₃₆ 24 h prior to re-feeding ($P<0.05$; Fig. 21). Thus, the half-life of the satiety effect exerted by PEG₄₀-FMS-PYY₃₋₃₆ was about 24 h, namely, 8-fold longer than that of unmodified PYY₃₋₃₆. In a control study, sc administration of PEG₄₀-FMS-Glycine ethyl ester (5 nmoles/mouse) 15 h before refeeding had no effect whatsoever on food intake as compared with saline (Fig. 21, right columns).

Example 32: Biological activity of (PEG₄₀-FMS)₂-hGH and PEG₄₀-FMS-hGH

Human growth hormone (hGH) is an essential pituitary hormone which regulates growth and development of peripheral tissues. hGH is an FDA-approved drug that is widely in use for replacement therapy in growth hormone deficient children. As valid for other non-glycosylated protein drugs of molecular mass lower than 50 kDa, hGH is cleared rapidly from the circulatory system having a $t_{1/2}$ value of 20-30 min in humans. Clearance takes place primarily via the kidney. The covalent attachment of PEG chains to hGH can substantially decrease clearance by glomerular filtration via the kidneys, and therefore elongate life-time *in vivo*. Several PEG chains must be introduced to hGH on order to appreciably elongate its

life-time *in vivo*. Pegylation often results in a drastic decrease in the biological or pharmacological potencies of peptides and proteins. This is especially valid for hGH owing to the relatively large surface area of site 1 and site 2 through which the hormone binds the first receptor and then the second receptor to form a
5 homodimeric receptor complex that initiates signaling.

The covalent attachment of PEG chains to hGH in a nonreversible fashion however, leads to a drastic loss in the biological potency of this hormone. For instance, Clark et al., 1996, have linked two to seven PEG₅₀₀₀ chains to the amino side chain moieties of hGH. The linkage of two PEG₅₀₀₀/hGH only already led to
10 ~90% loss in the biological potency of hGH. About five such PEG₅₀₀₀ chains had to be linked to the protein for increasing circulating half-life substantially. Such (PEG₅₀₀₀)₅ hGH conjugates however had less than 0.1% the biological potency of hGH.

Two PEG-FMS-hGH conjugates have been prepared according to the
15 invention (Examples 24-25). In fact, hGH contains several conjugatable amino functions. However, in view of the pegylation reaction's reversibility according to the present invention and regeneration of the native peptide and protein hormones in physiological condition, this point deserves only minor consideration.

20 **32(i) Receptor-binding capacity of (PEG₄₀-FMS)₂-hGH and PEG₄₀-FMS-hGH**

The hGH displacement assay was performed as previously described (Tsushima et al., 1980). (PEG₄₀-FMS)₂-hGH, prepared by linking two PEG₄₀-FMS moieties to hGH, has an effective molecular weight of about 120 kDa and exhibits 9±2% the receptor binding potency of the native hGH. Receptor binding capacity is
25 regenerated upon incubation at 37°C in normal rat serum or in 0.1M phosphate buffer (pH 8.5) at 37°C with a t_{1/2} value of ~20 hrs, reaching 70-80% the native-receptor binding capacity following 50 hours of incubation (not shown).

32(ii) *The hydrolysis rate and reaction order of PEG₄₀-FMS-hGH*

The hydrolysis rate and reaction order of PEG₄₀₀₀₀-FMS-hGH were evaluated at pH = 8.5, 37°C. The release of hGH was monitored by RP-HPLC and was determined to be a first order reaction (Table 3, Fig. 22). *In vitro*, PEG₄₀₀₀₀-FMS-hGH exhibits no receptor binding affinity. Upon incubation, recognition of the released hGH by the native hGH receptor was preserved (Fig. 22). Human growth hormone was released from the conjugate in a slow homogenous fashion, with $t_{1/2}$ of 11.8 h (Table 3, Fig. 23).

10 **Example 33: PEG₄₀-FMS-ANP undergoes time-dependent hydrolysis at physiological conditions**

Atrial natriuretic peptide (ANP) is a 28-mer amino acid non-lysine containing peptide which exerts natriuretic, diuretic and vasorelaxant actions. It plays an important role in the body's blood volume and blood pressure homeostasis. ANP has a very high affinity for its receptor sites (10 pm), and is therefore cleared very rapidly ($t_{1/2}$ ~0.5 min) by receptor and protease-mediated events. ANP is therefore not suitable as a drug for subcutaneous administration for regulation of blood pressure homeostasis in humans. The covalent attachment of a PEG₄₀-chain to the α -amino moiety of ANP generates a conjugate which is fully devoid of receptor binding affinity (data not shown).

PEG₄₀-FMS-ANP, prepared by linking PEG₄₀-FMS to the α -amino side chain of ANP, is TNBS negative. Upon incubation at pH 8.5 and 37°C, this conjugate undergoes spontaneous hydrolysis with a $t_{1/2}$ value of 23±2 hrs, generating the parent ANP following 50 hours of incubation (Table 5). Preliminary measurements in rats *in vivo* revealed that SC-administered PEG₄₀-FMS-ANP has a 60-fold increase in serum half-life, in comparison to native ANP. The conjugate is fully protected from receptor-mediated degradation prior to the fall-off of the PEG₄₀-FMS chain from ANP by hydrolysis.

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Table 1. Chemical features of MAL-FMS-NHS

| Characteristic | Numerical value |
|--|--|
| Solubility in aqueous buffer (pH 7.2) | >10 mg/ml |
| Mass spectra data ^a | |
| Calculated ESMS | 583 Da |
| Found ESMS for [M+H] ⁺ | 584.52 Da |
| Found ESMS for [M+Na] ⁺ | 606.47 Da |
| Retention time (analytical HPLC) ^b | 2.65 min |
| Molar extinction coefficient at 280nm ^c | 21,200±200 mole ⁻¹ cm ⁻¹ |
| Molar extinction coefficient at 320nm ^c | 16,100±150 mole ⁻¹ cm ⁻¹ |

^a Mass spectra were determined using the electrospray ionization technique

^b The HPLC column used is a Chromolith column; (C₁₈); linear gradient of 10-100% of solvent B (3 ml/min).

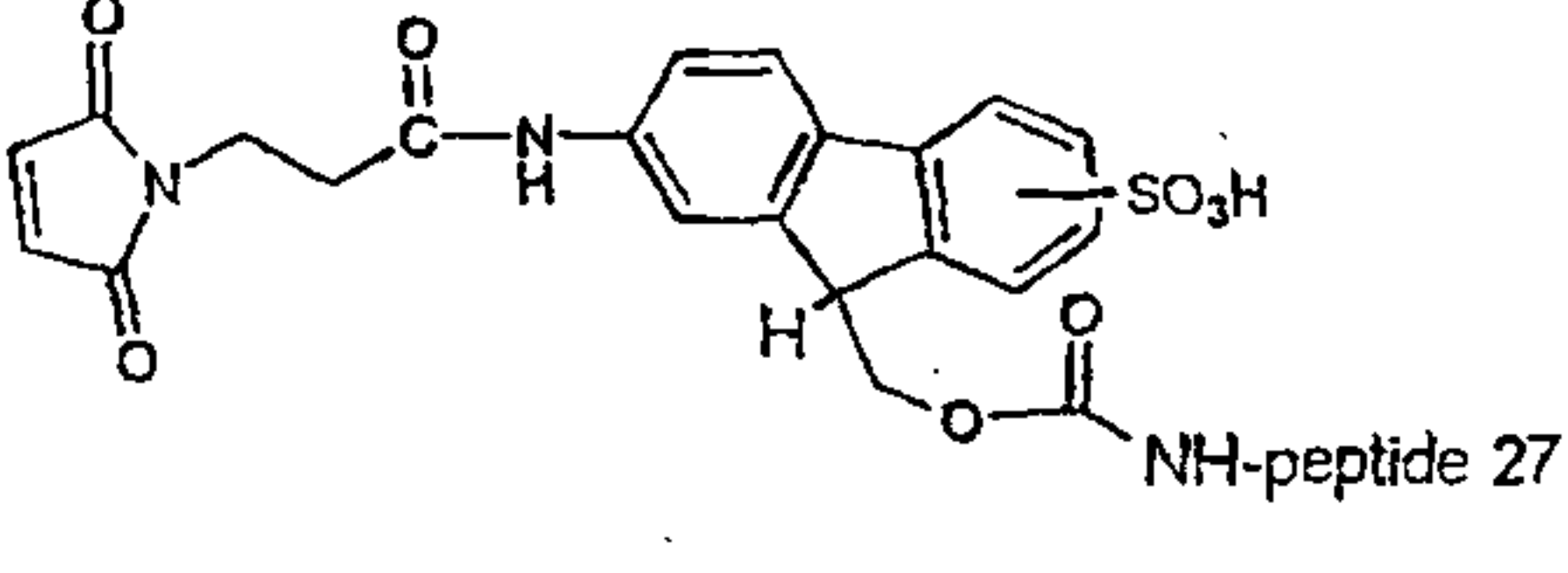
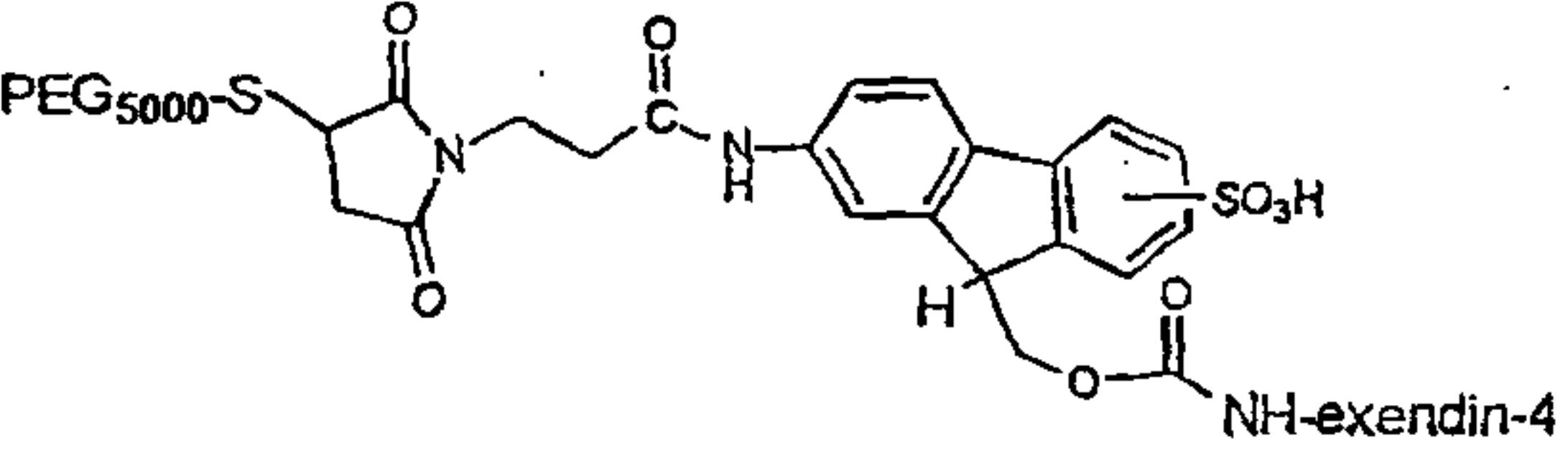
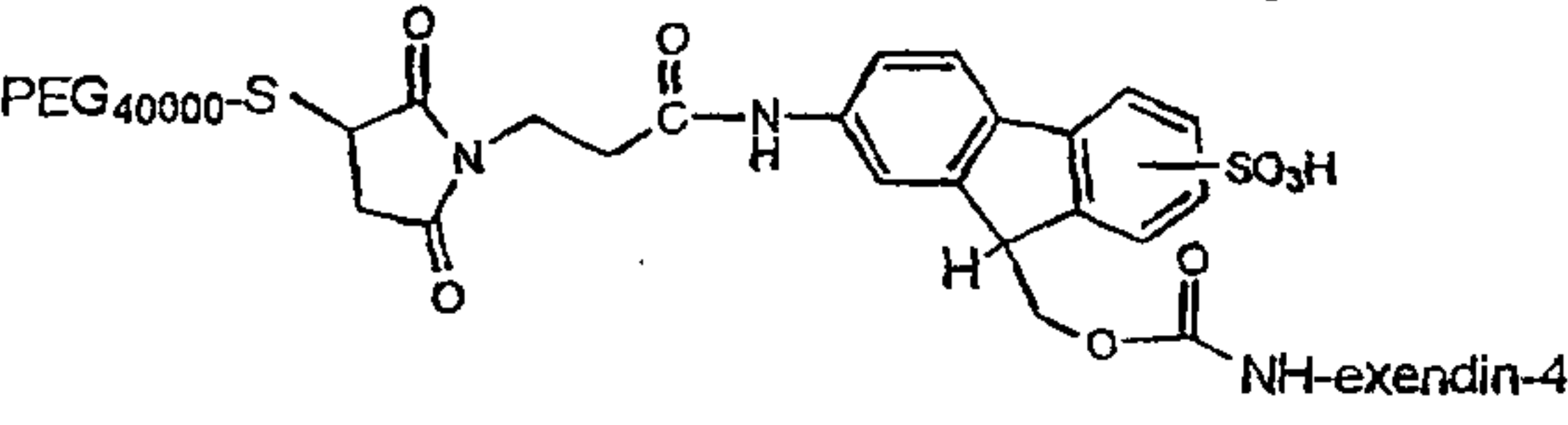
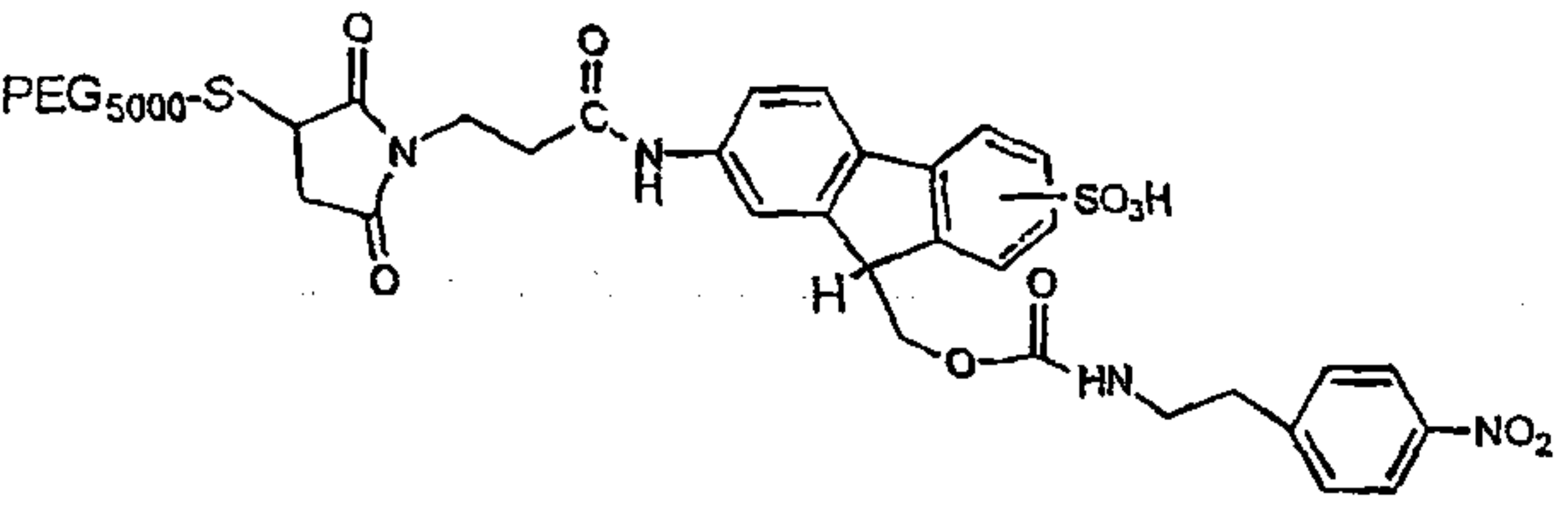
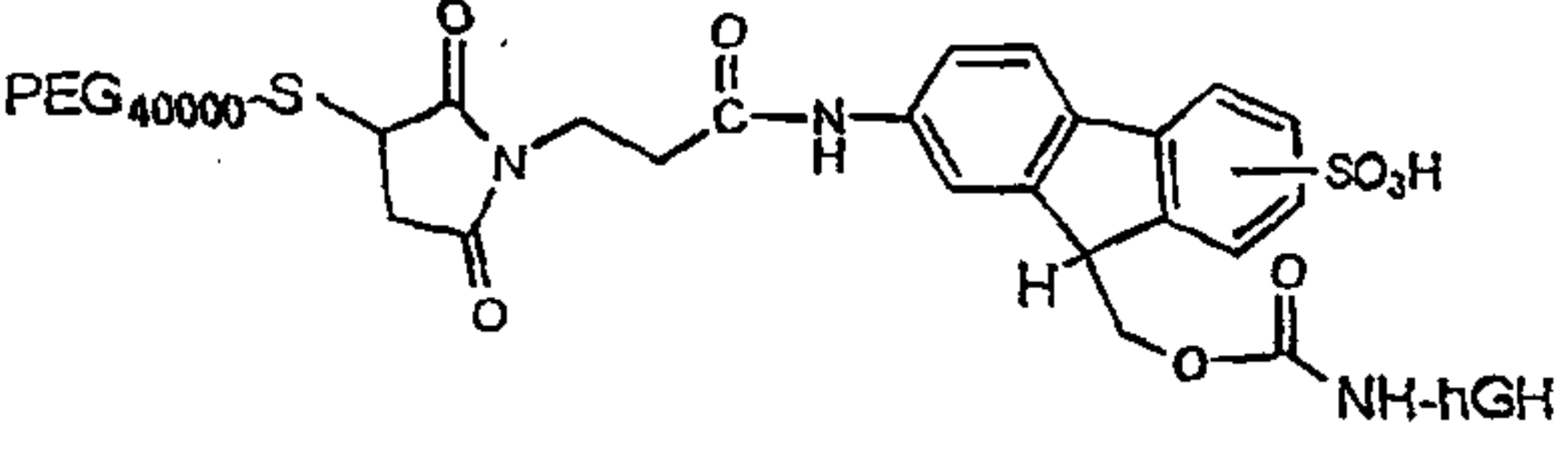
^c Based on the absorbance at 280 and 301 nm in PBS, pH 7.2, with compound concentration determined by quantitating the MAL-function with excess GSH and DTNB.

Table 2. Chemical features of PEG₄₀-FMS-IFN α 2

| Characteristic ^a | Numerical Value |
|--|----------------------------------|
| Absorbance at 280nm ^{b,c} | $\epsilon_{280} = 39270 \pm 100$ |
| Mass spectra ^d | |
| PEG-FMS-IFN α 2 ^e , calculated | 63569 daltons |
| PEG-FMS-IFN α 2 ^e , measured | 63540 daltons |
| Retention time (analytical HPLC) ^f | 43 \pm 0.5 min. |
| Solubility in aqueous buffer, pH 7.4 | >20 mg/ml |

- (^a) For characterization, IFN α 2-FMS-MAL was dialyzed against H₂O prior to linking PEG₄₀-SH. The final product was filtered through a centricon having a cut-off value of 50 kDa. These procedures remove free MAL-FMS-NHS and any residual native IFN α 2, or IFN α 2-FMS-MAL that has not been linked to PEG₄₀-SH.
- (^b) Determined by UV spectroscopy. Derivative concentration was determined by acid hydrolysis of a 20 μ l aliquot followed by amino acid analysis, calculated according to aspartic acid (14 residues), alanine (9 residues) and isoleucine (8 residues).
- (^c) Native IFN α 2 absorbs at 280 nm with $\epsilon_{280} = 18070$ (30).
- (^d) Mass spectra were determined by using MALDI - TOF mass spectroscopy.
- (^e) Calculated mass is obtained by the additive masses found for native IFN α 2 (19278 daltons); for PEG₄₀-SH (43818 daltons) and for the spacer molecule following conjugation (473 daltons).
- (^f) Native IFN α 2 elutes under identical analytical HPLC procedure with retention time = 33.9 min.

Table 3. Structures and half-life time of PEG-FMS conjugates.

| | Structure | k (h^{-1}) ^b | $t_{1/2}$ (h) ^c |
|---|--|--------------------------------------|----------------------------|
| Mal-FMS-Peptide 27 ^a |  | 0.082 | 8.4 |
| PEG ₄₀₀₀₀ -FMS-exendin-4 ^d |  | 0.058 | 11.9 |
| PEG ₅₀₀₀ -FMS-exendin-4 |  | 0.050 | 13.8 |
| PEG ₅₀₀₀ -FMS-4-nitrophenethylamine |  | 0.074 | 9.4 |
| PEG ₄₀₀₀₀ -FMS-human growth hormone (hGH) ^d |  | 0.059 | 11.8 |

^a Rate of hydrolysis was followed by trinitrobenzene sulfonic acid assay

^b k is the slope constant derived from the plot of \ln [PEG-FMS conjugate] against time (h)

5 ^c $t_{1/2}$ was determined from the formula $t_{1/2} = \ln 2/k$.

^d plot of hydrolysis can be found as Fig. 23.

Table 4. Receptor binding capacity of mono- and bis-PEG-FMS-IFN- α 2 conjugates prior to, and following, incubation with ifnar-2-EC.

| Derivative designation | Mole PEG ₄₀ -FMS per mole IFN α 2 | Receptor binding capacity ^(a) % | Receptor binding capacity following 50 h of incubation at pH 8.5, 37°C % ^(b) | t _{1/2} of regenerating receptor binding capacity (h) |
|---|---|--|---|--|
| Native IFN α 2 | - | 100 | 97 \pm 2 | - |
| (PEG ₄₀ -FMS) ₁ -IFN α 2 | 1.0 | 9 \pm 1 | 95 \pm 4 | 9 \pm 1 |
| (PEG ₄₀ -FMS) ₂ -IFN α 2 | 1.9 | 0.4 \pm 0.05 | 92 \pm 3 | 24 \pm 3 |

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(a) Receptor binding capacity toward immobilized ifnar-2-EC was assessed by the reflectometric interference spectroscopy procedure-RIFS.

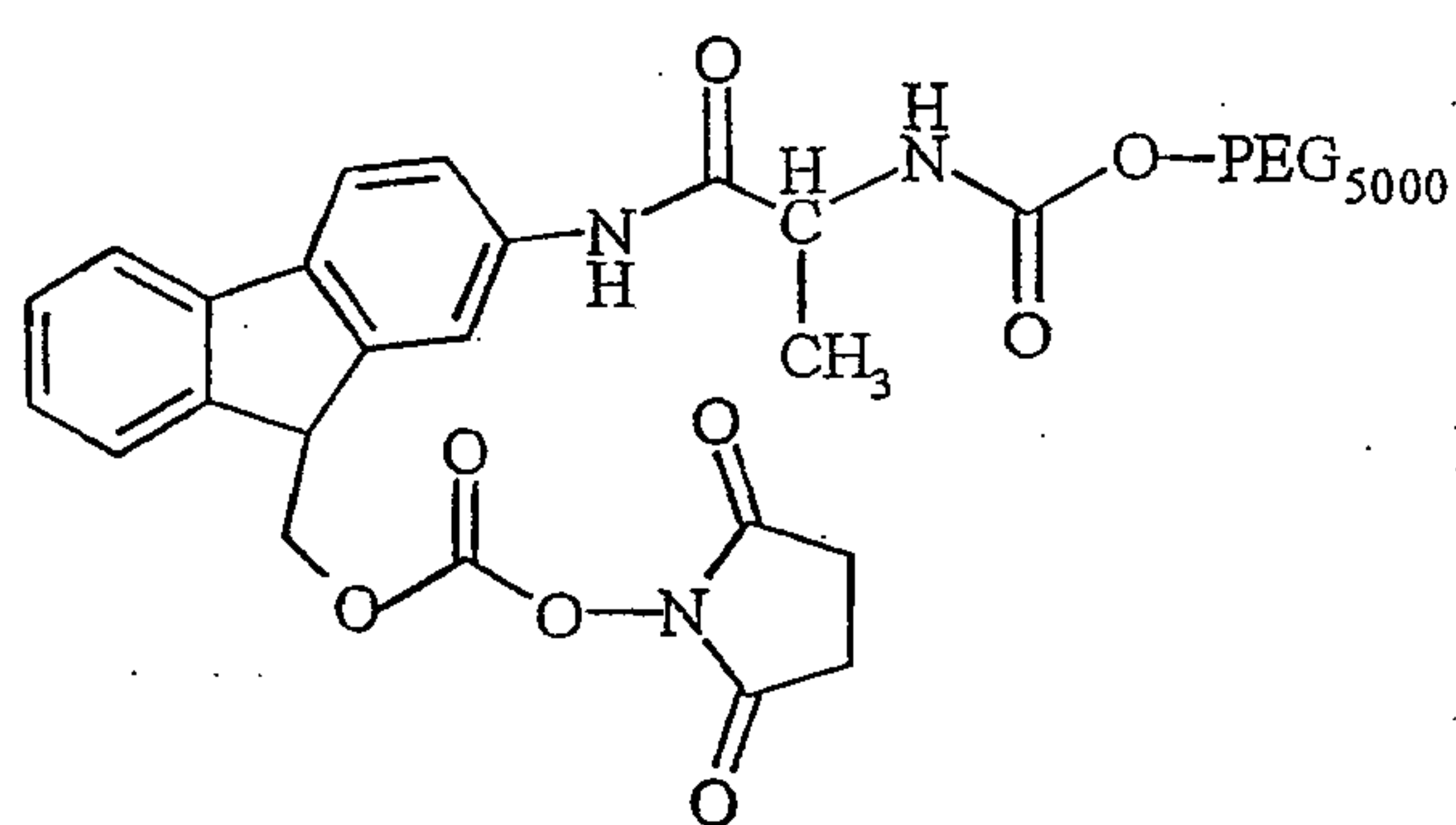
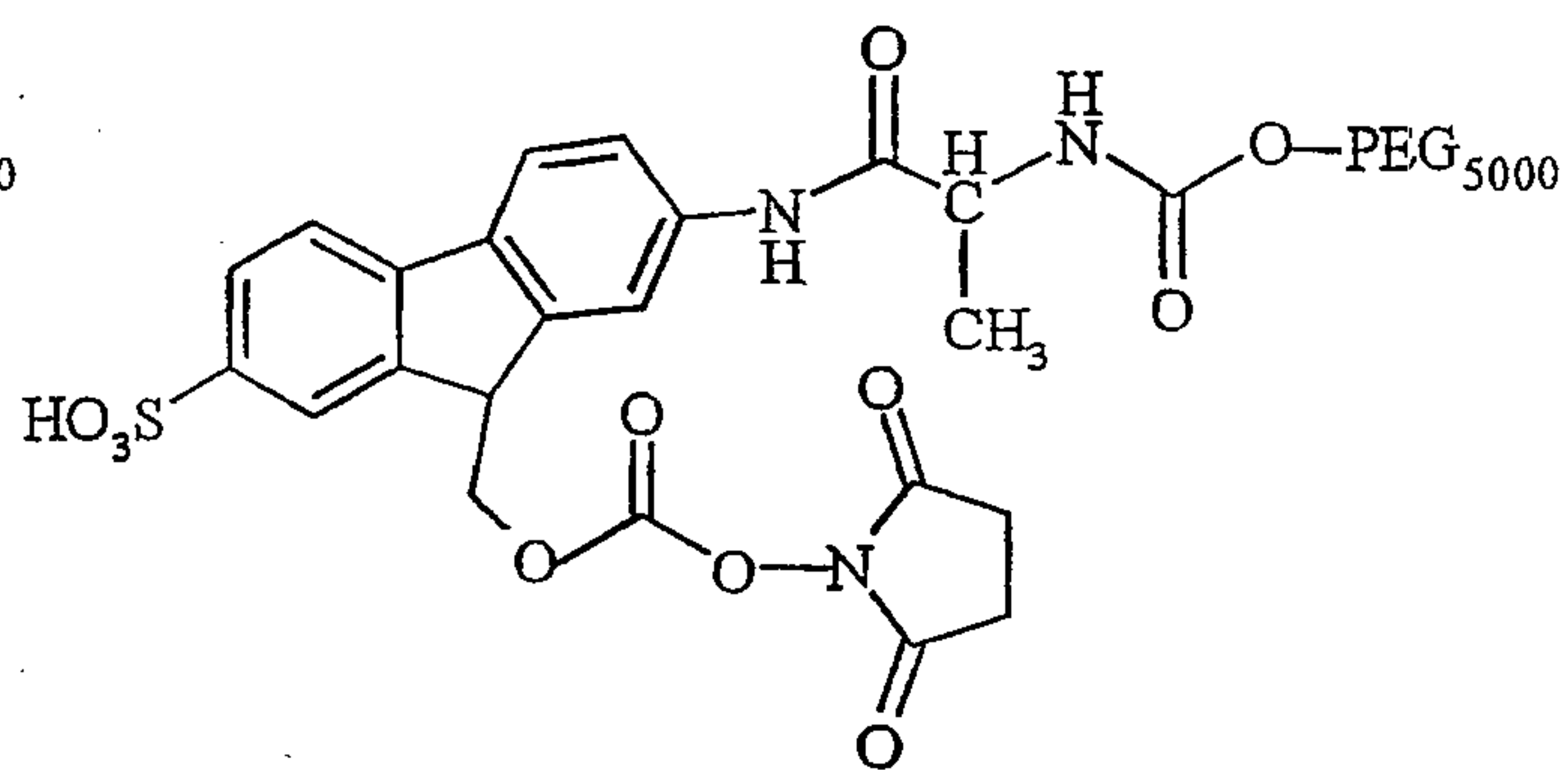
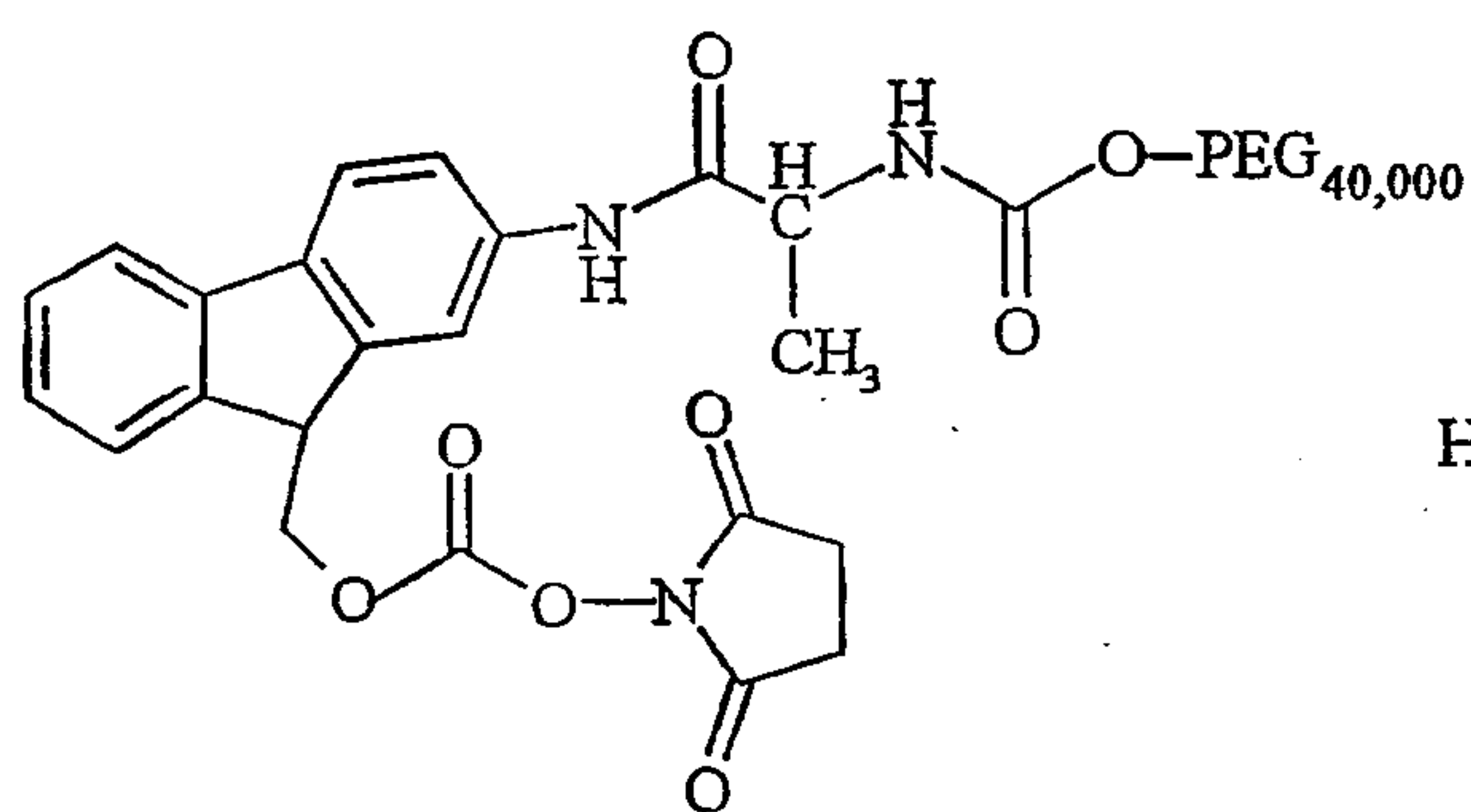
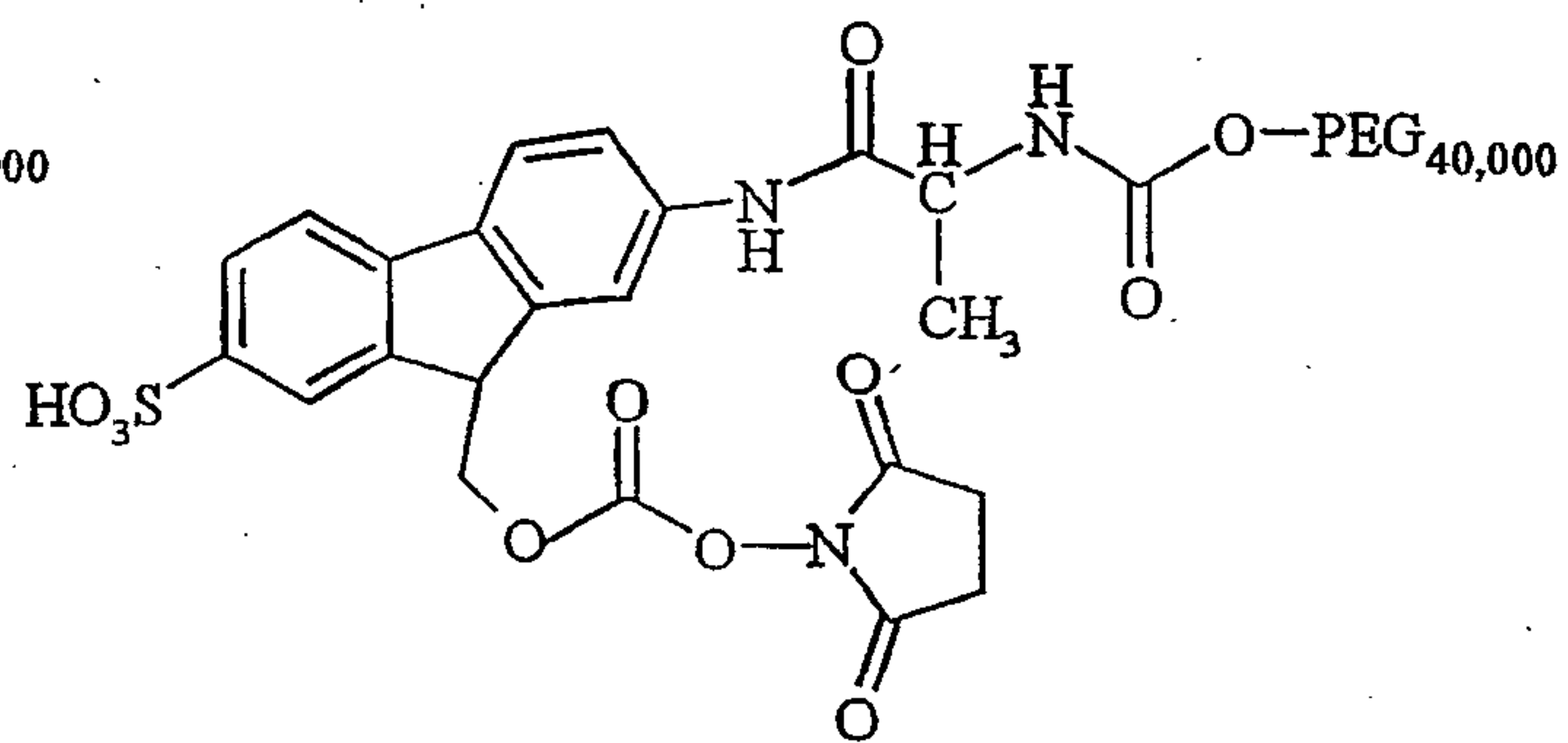
Incubation was performed in 0.1M phosphate buffer pH 8.5, containing 0.5% BSA.

Table 5. Rate of hydrolysis of PEG₄₀-FMS-ANP at pH 8.5, 37°C.

| Time of Incubation (pH 8.5, 37°C) hours | % hydrolyzed |
|--|--------------|
| 0 | 0 |
| 3 | 7 |
| 6 | 18 |
| 10 | 27 |
| 15 | 40 |
| 23 | 50 |
| 33 | 75 |
| 43 | 87 |
| 50 | 100 |

Scheme 1: *Precursors 1-4*

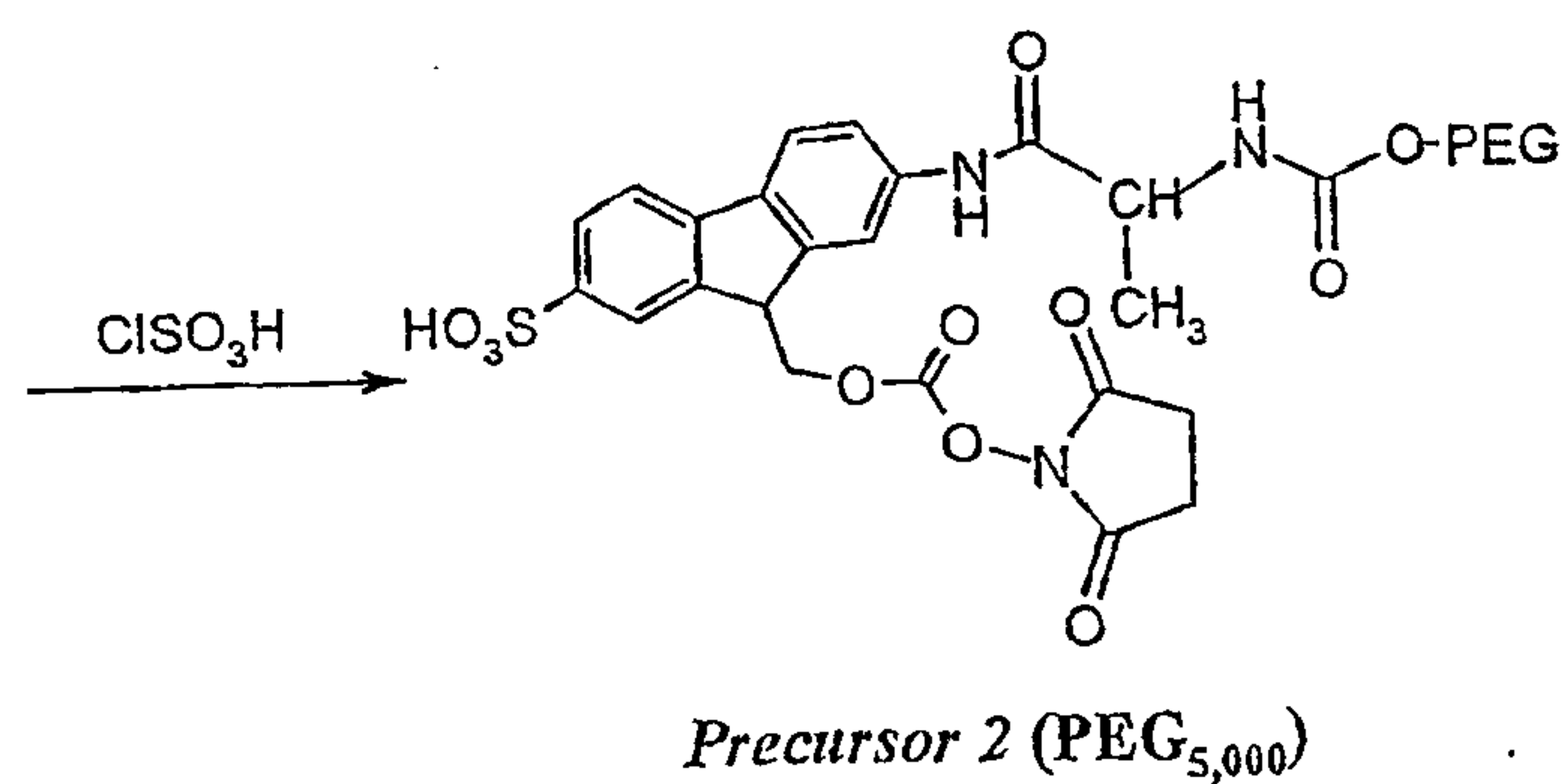
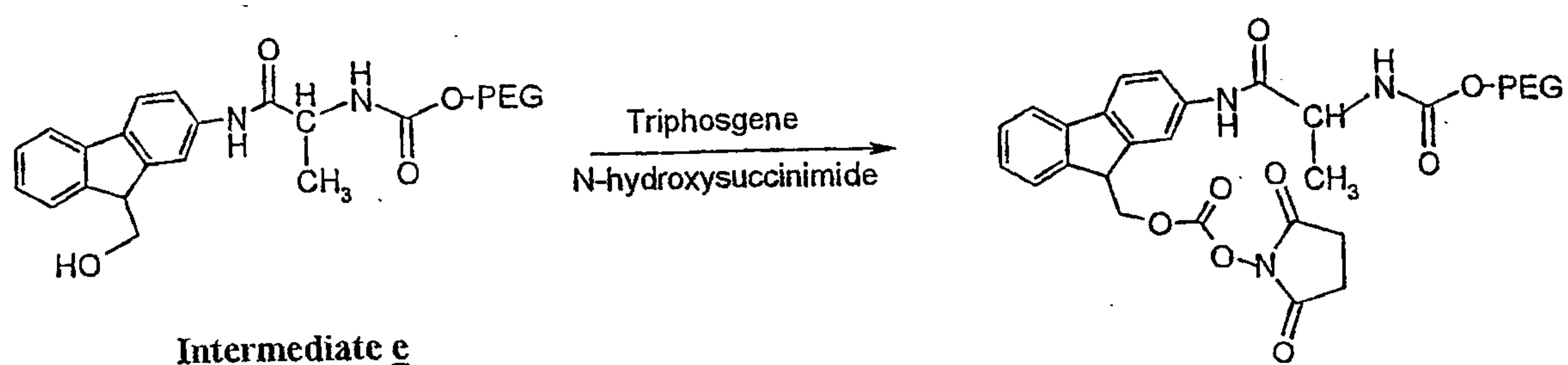
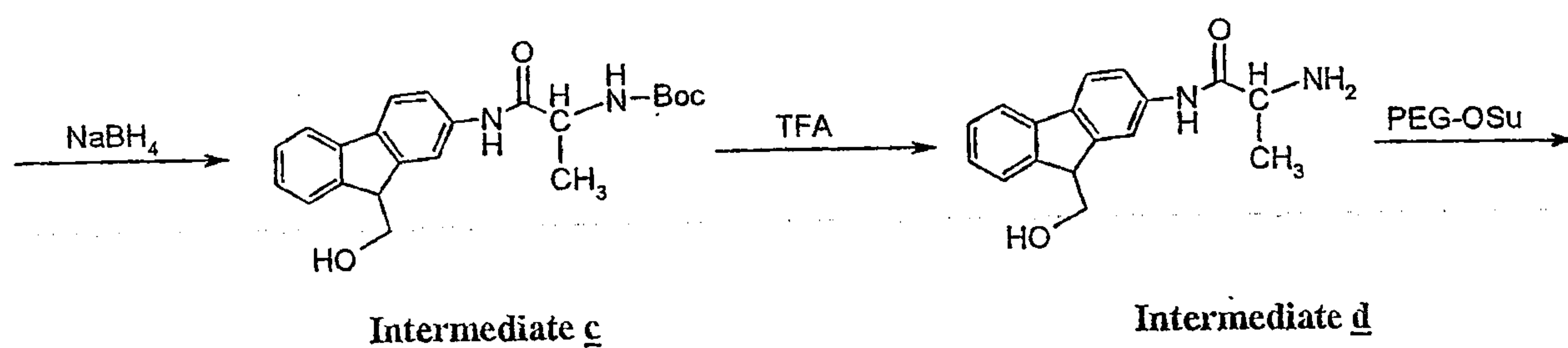
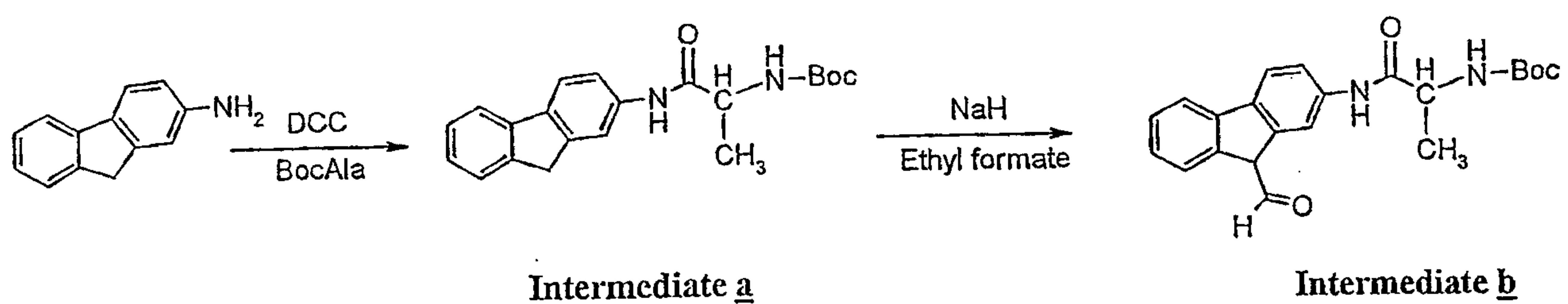
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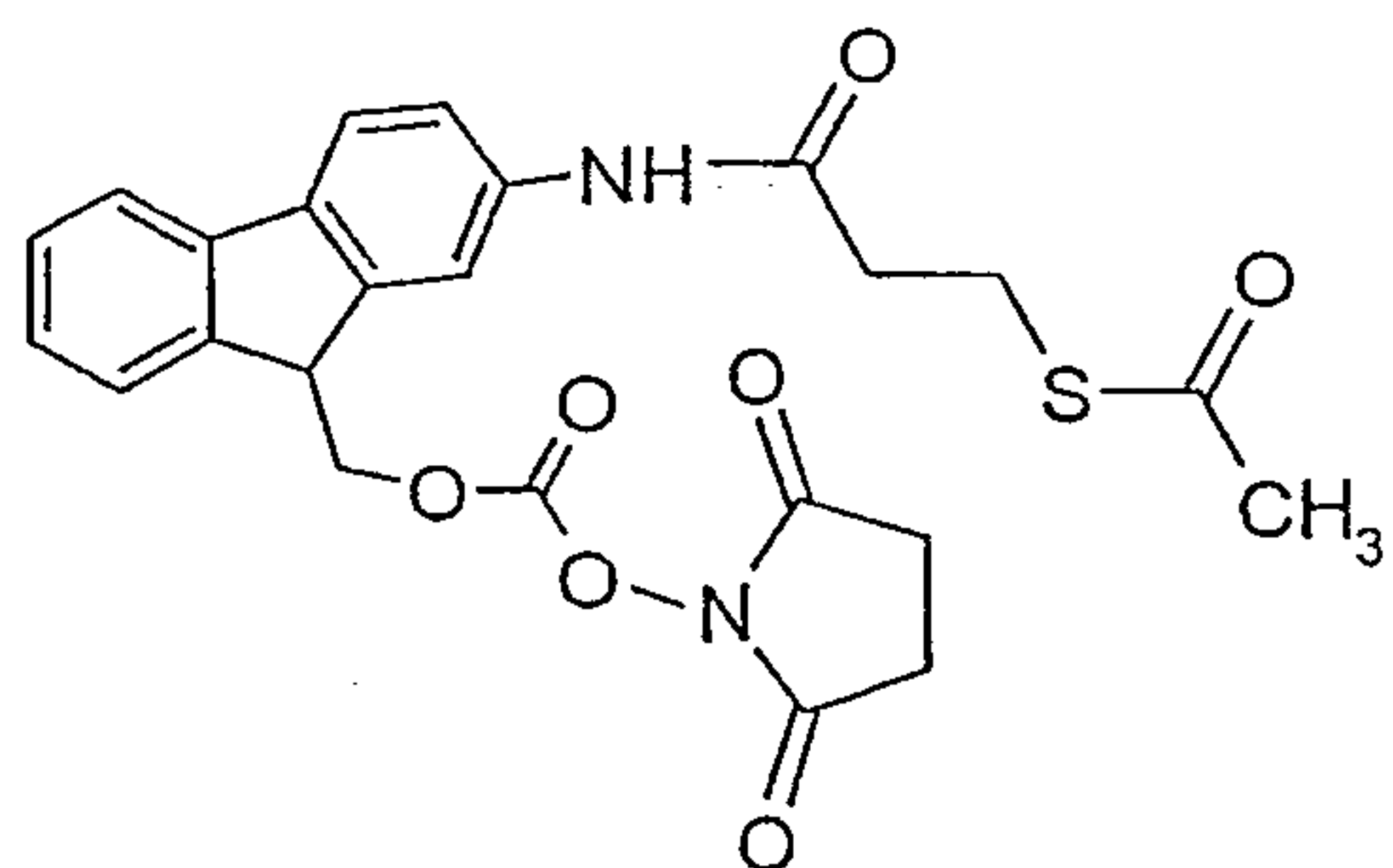
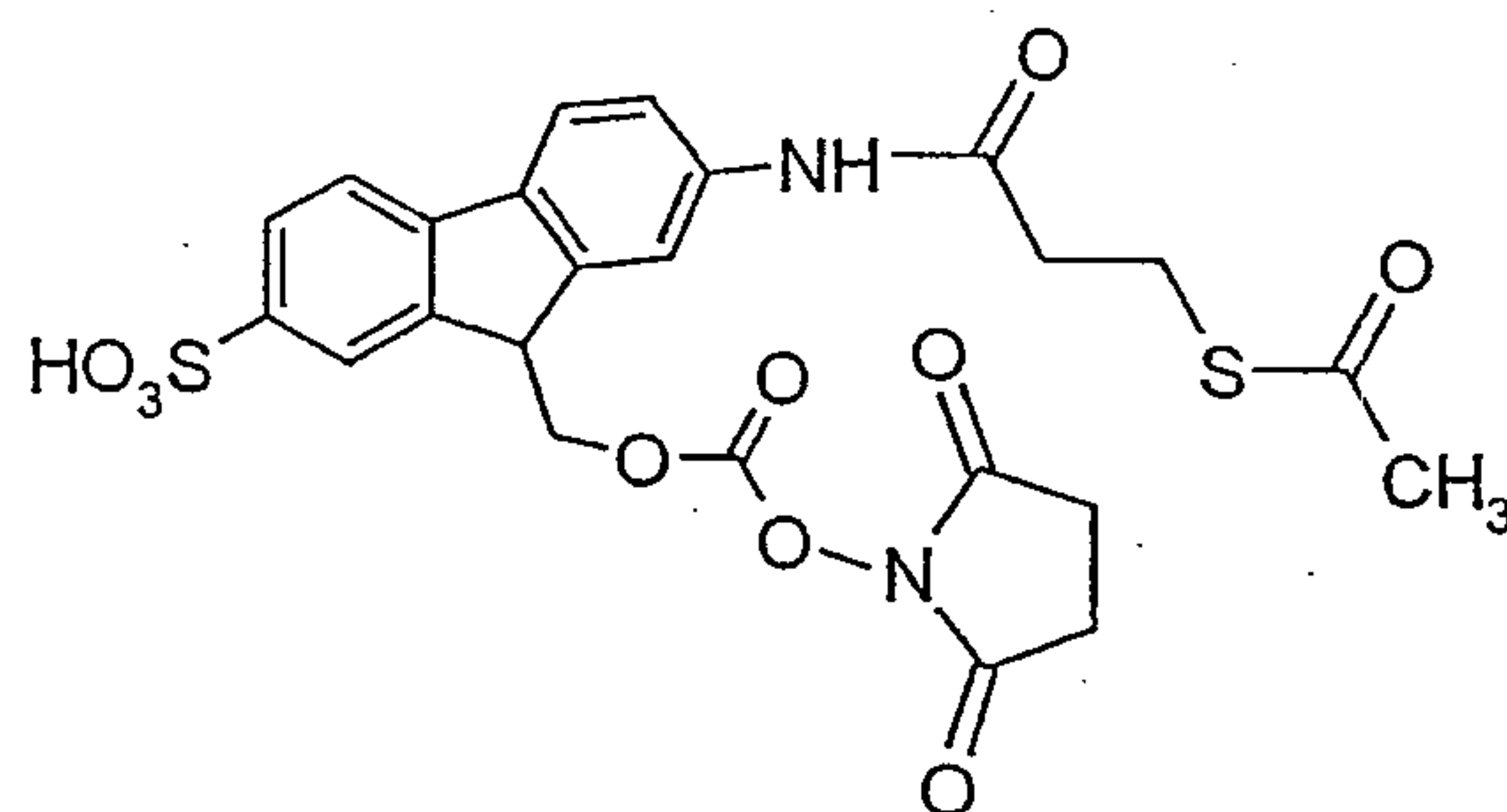
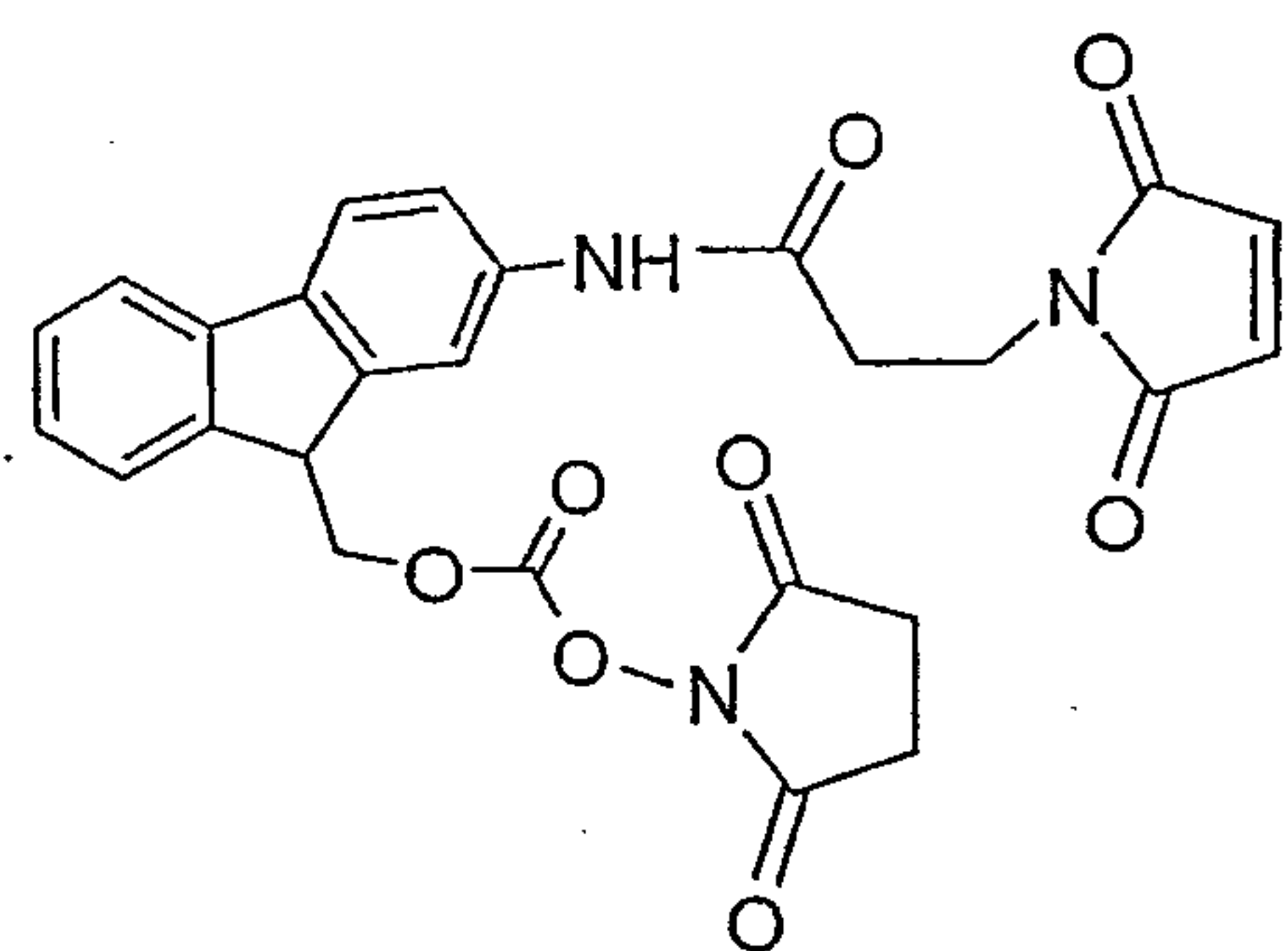
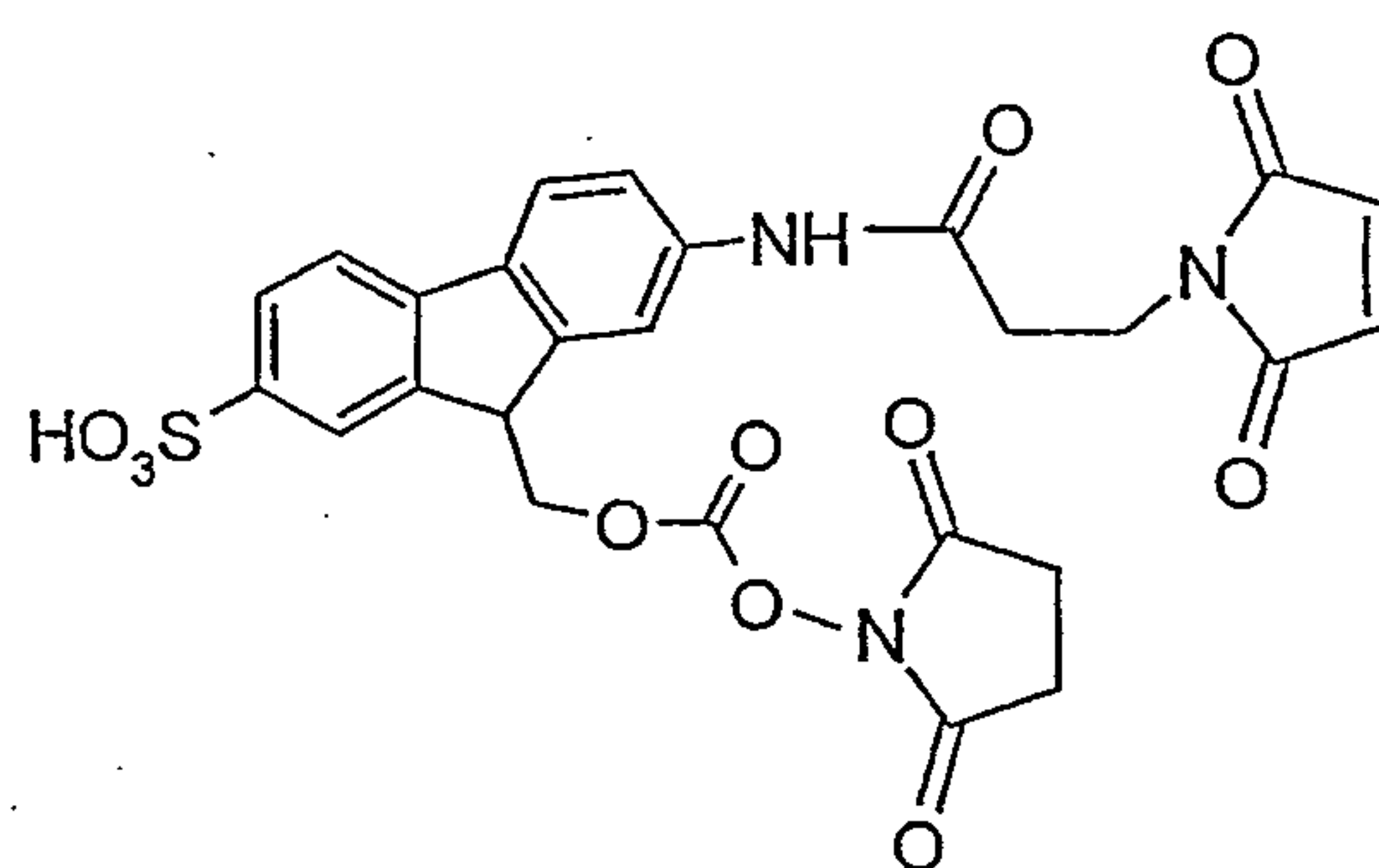
*Precursor 1**Precursor 2**Precursor 3**Precursor 4*

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Scheme 2: Synthesis of *Precursors 1-4*

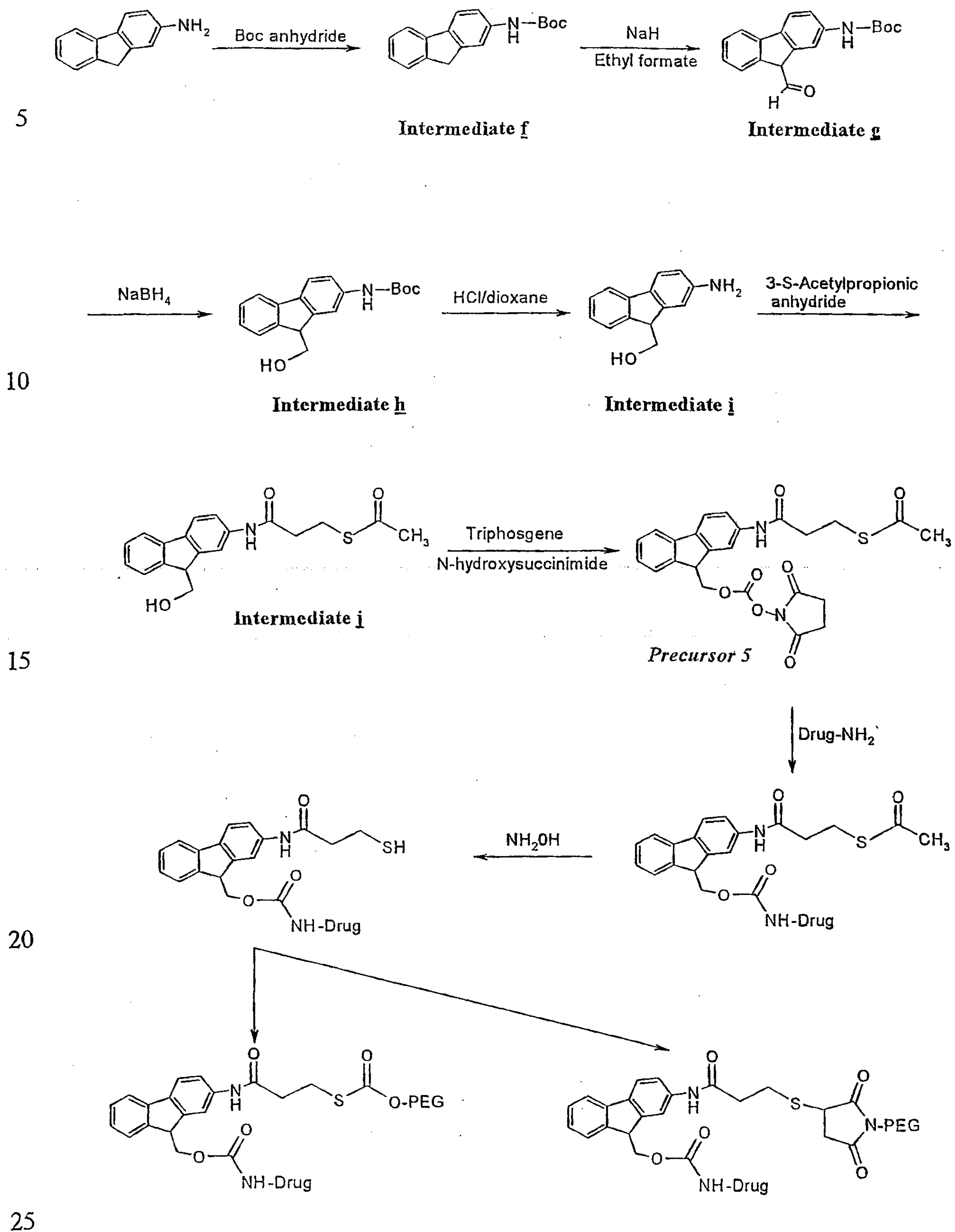
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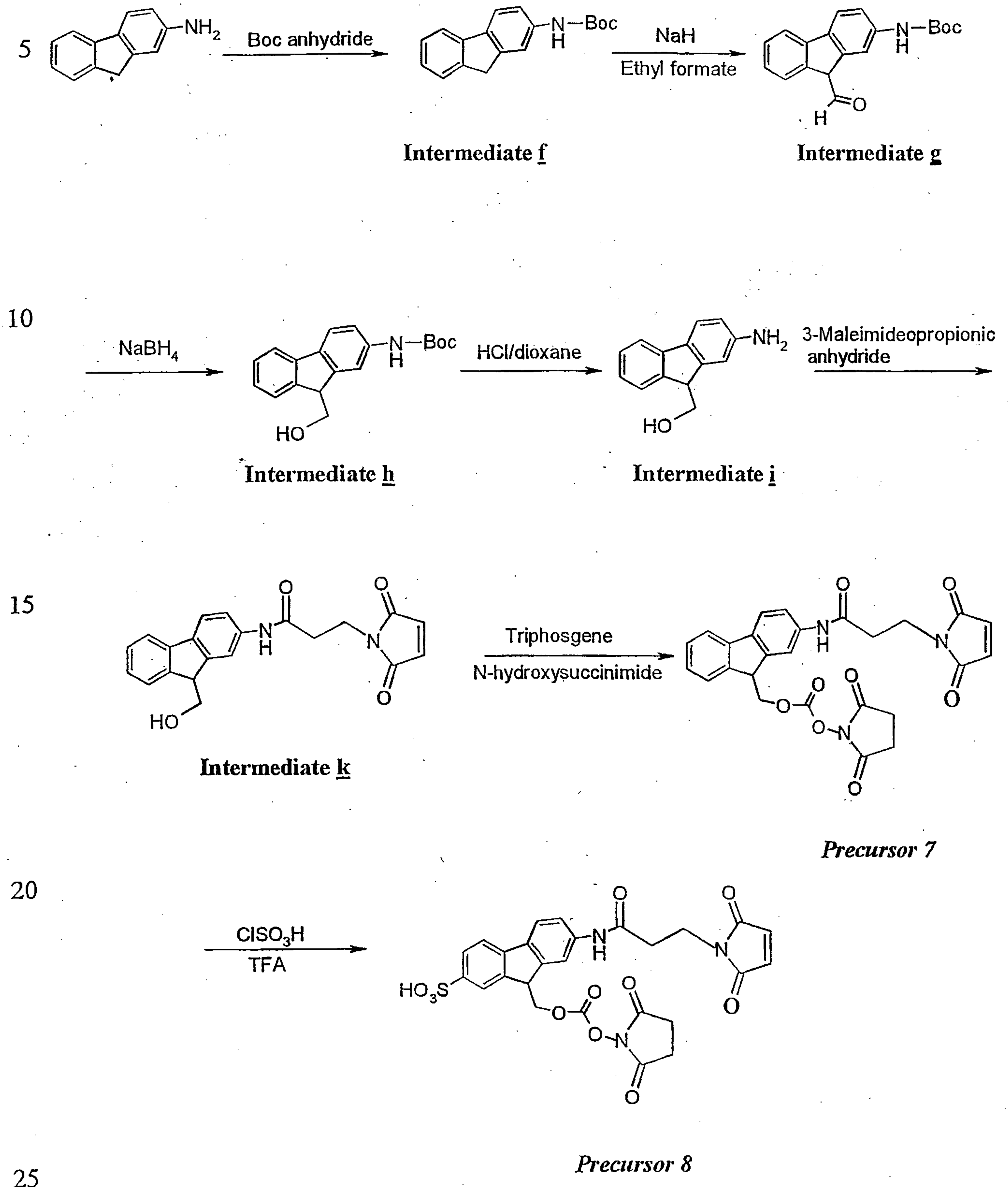


Scheme 3: *Precursors 5-8**Precursor 5**Precursor 6**Precursor 7**Precursor 8*

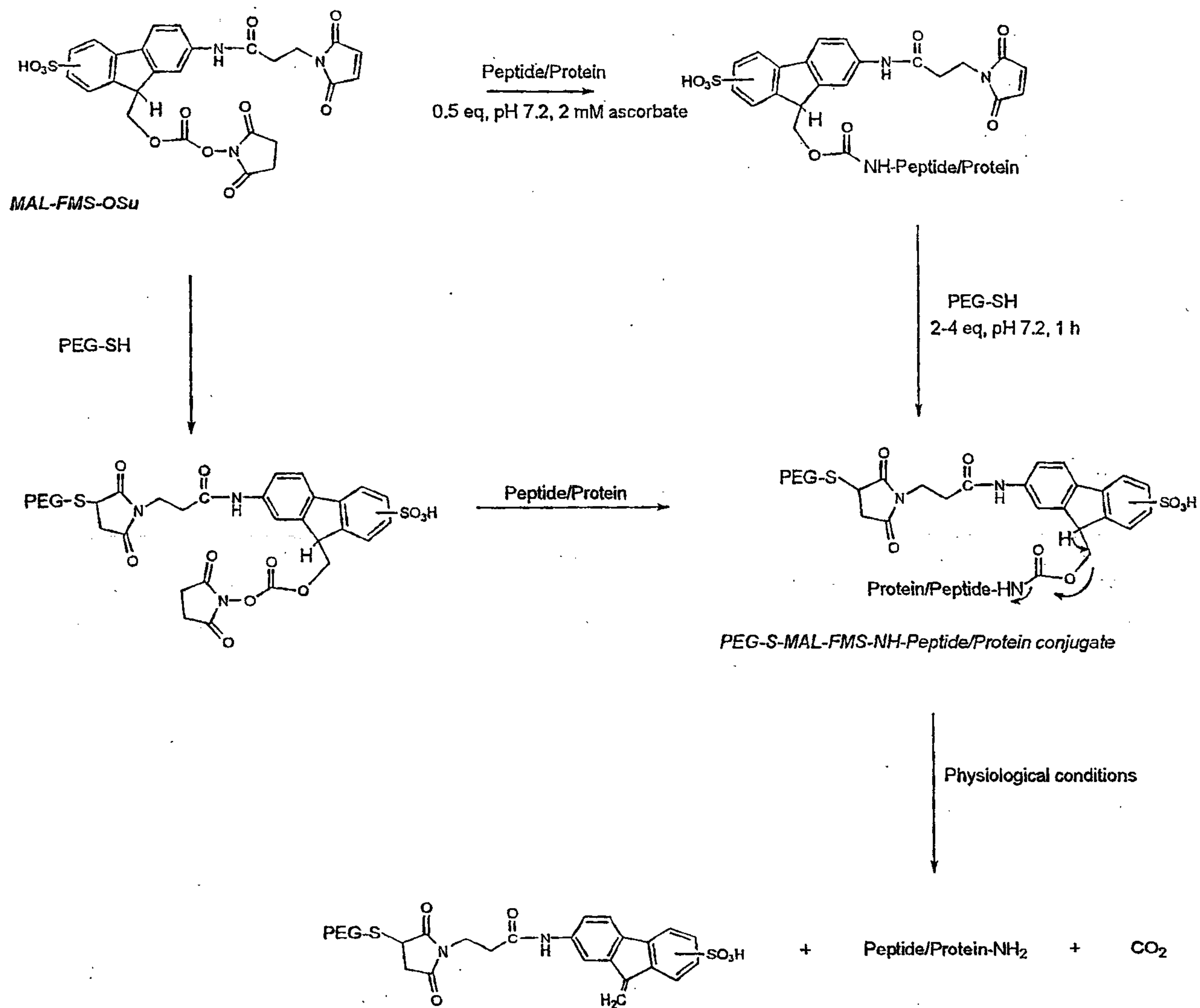
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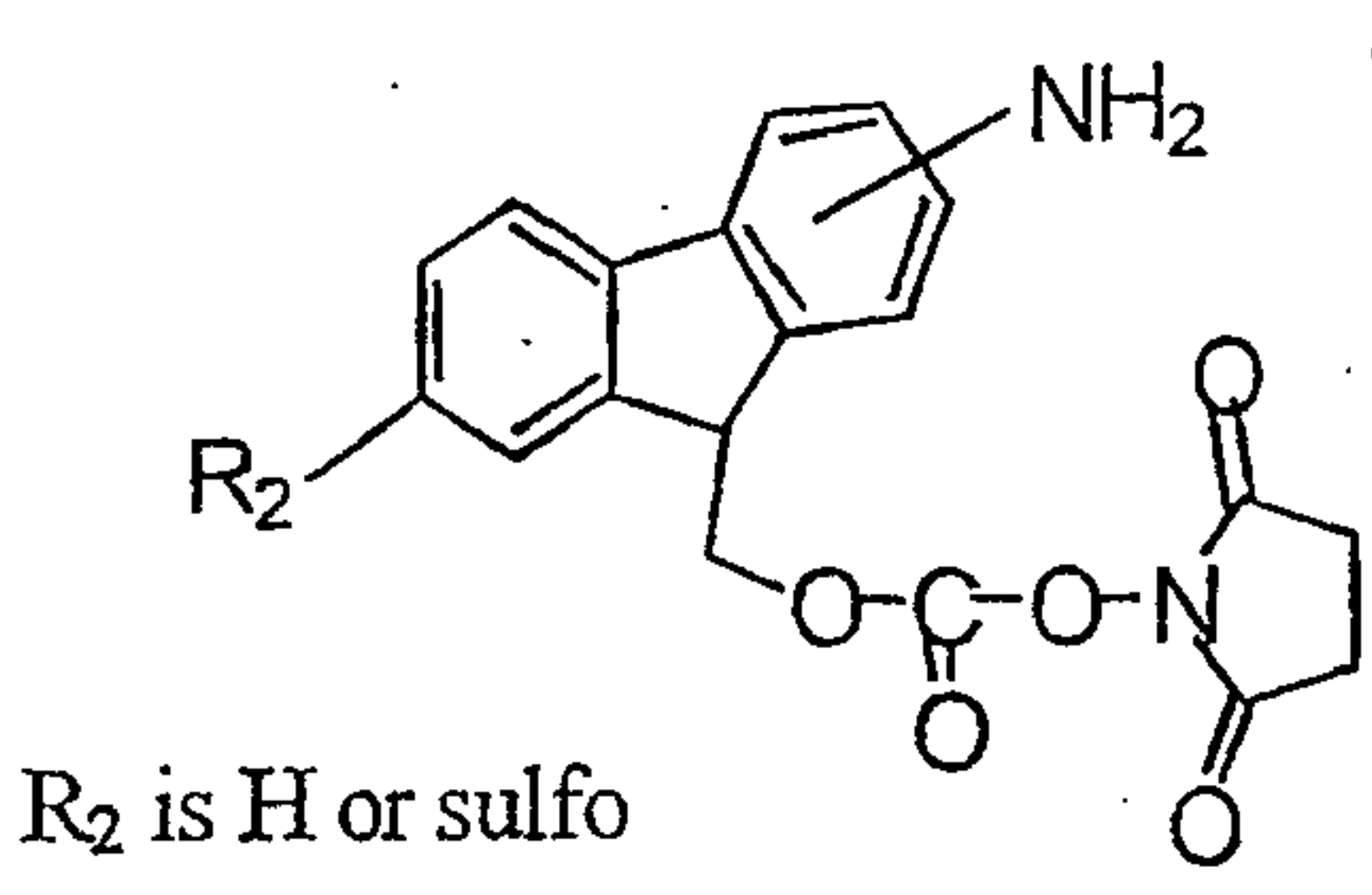
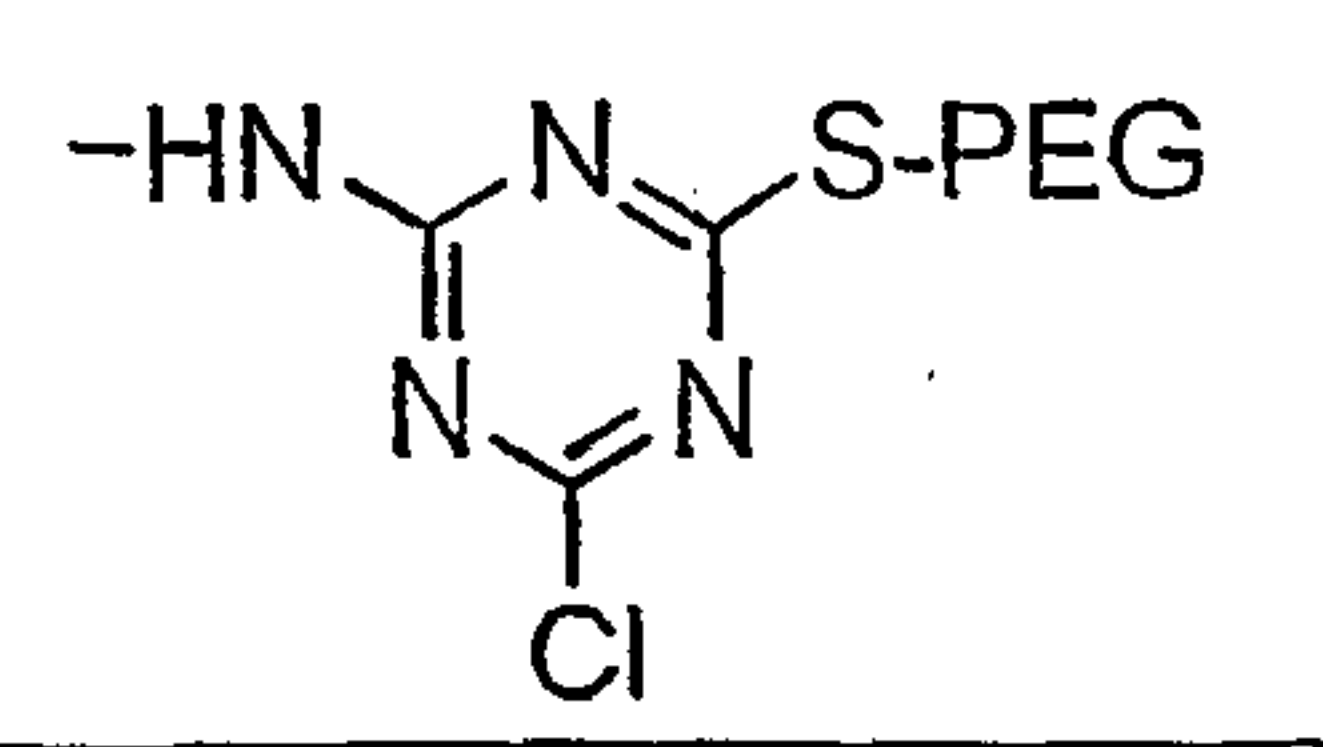
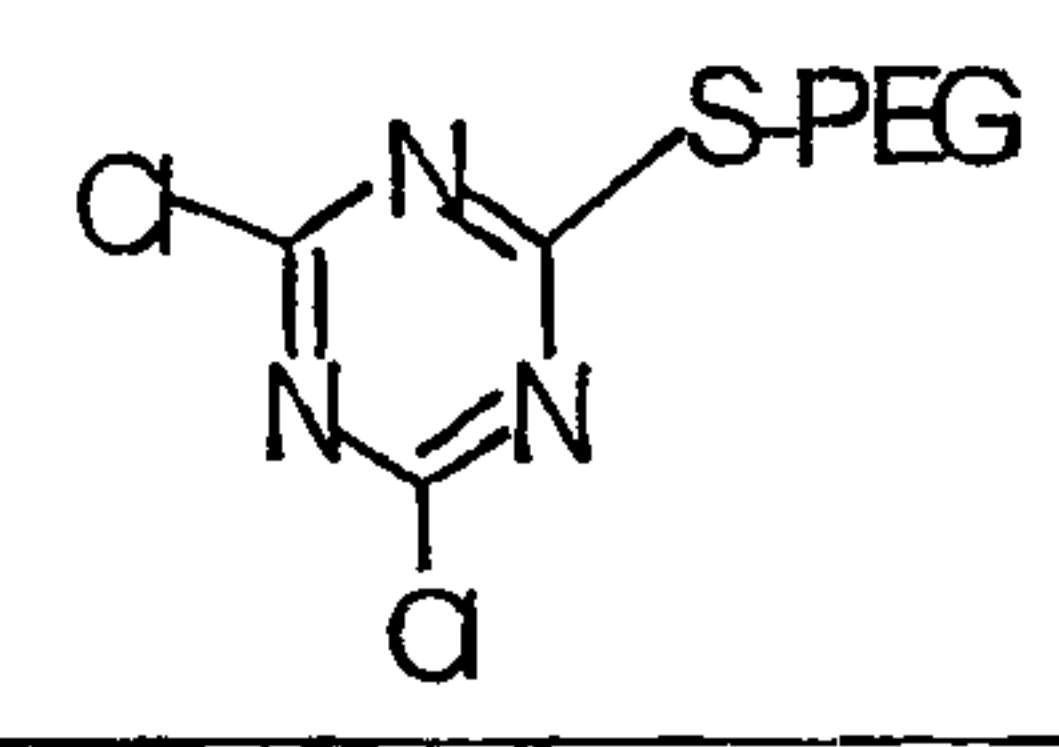
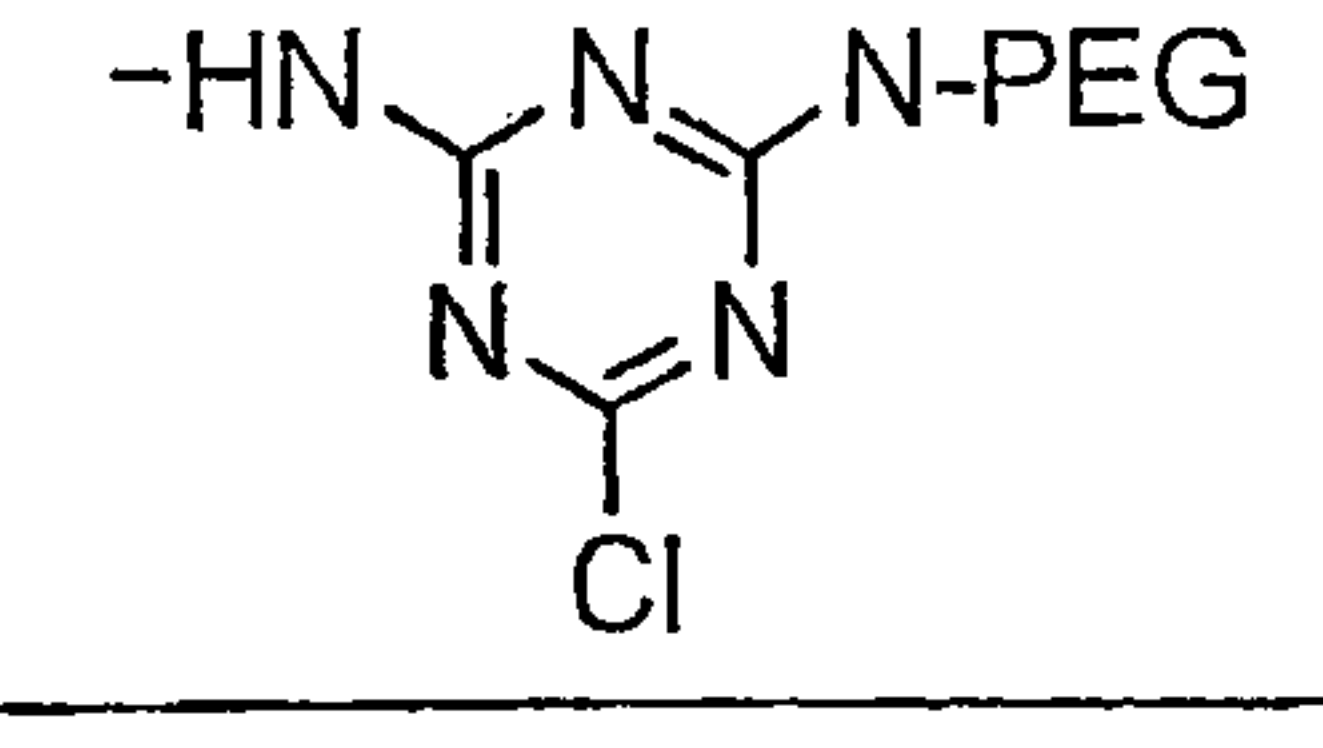
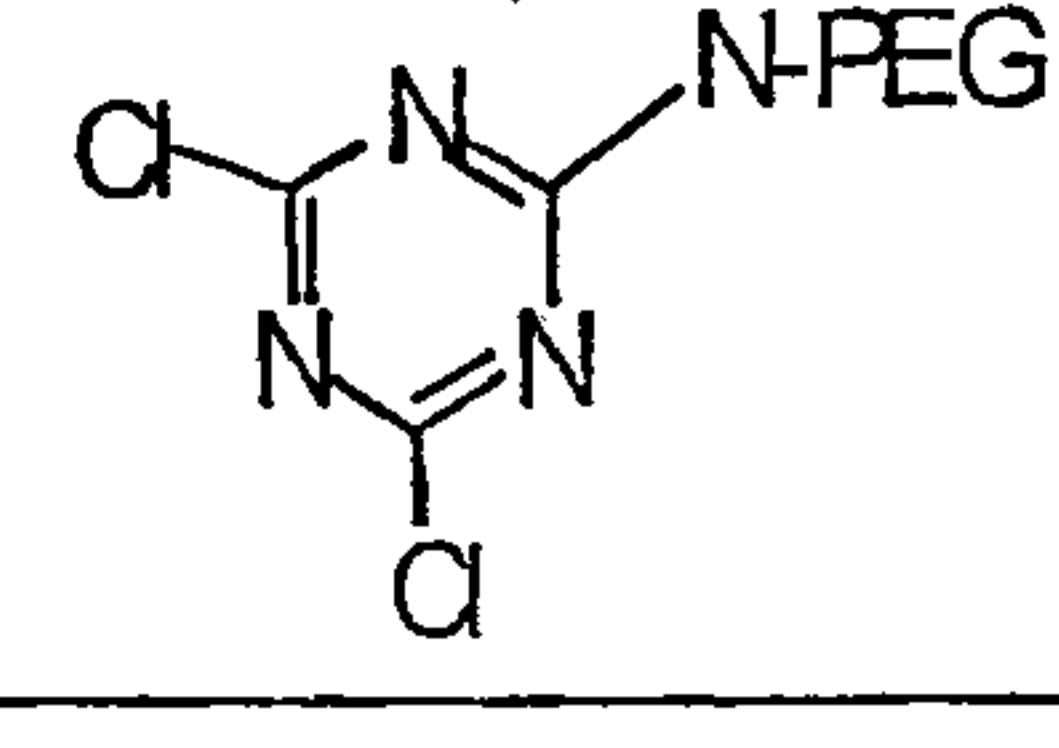
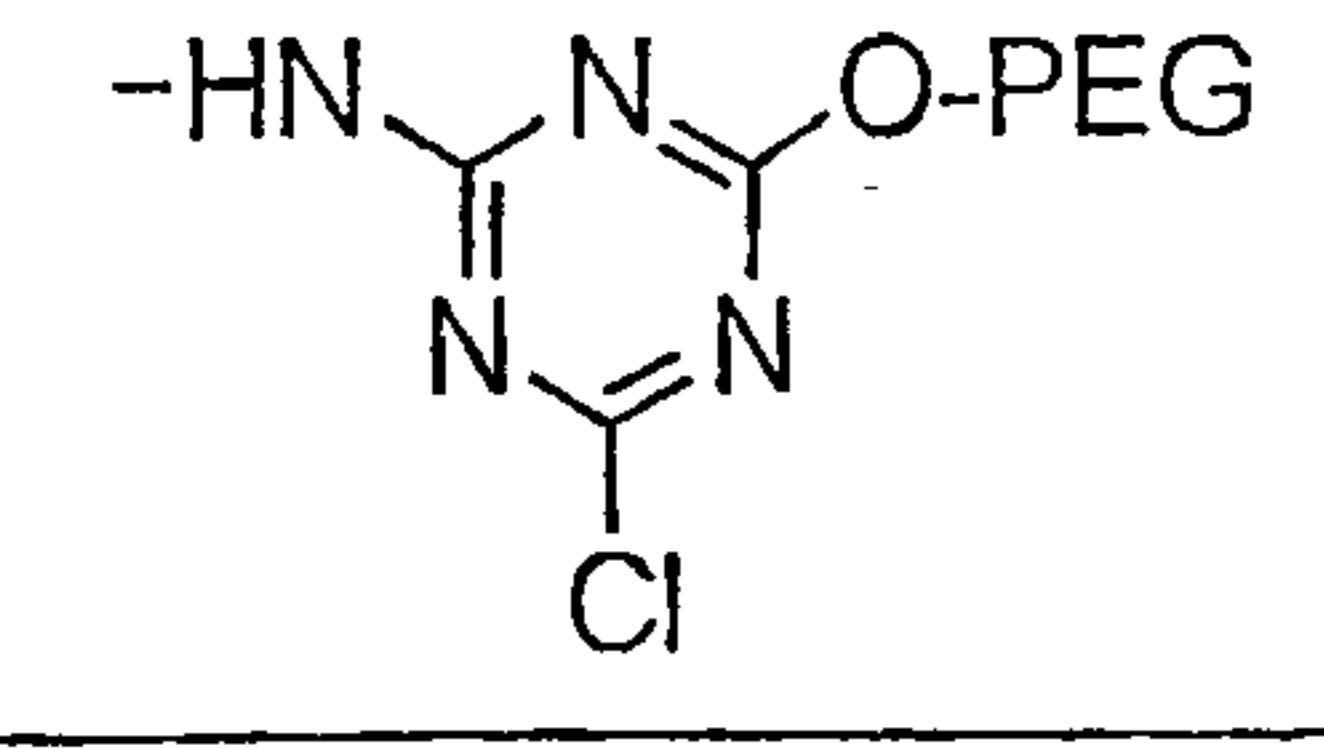
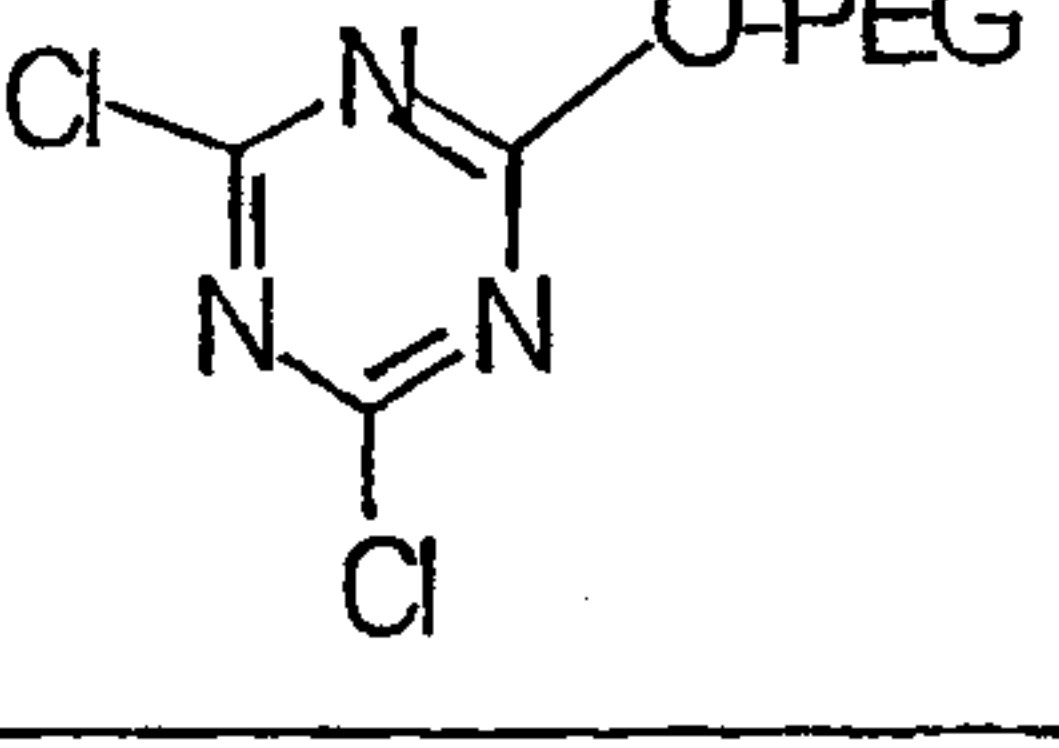
Scheme 4: Synthesis of *Precursor 5*

Scheme 5: Synthesis of *Precursors 7 and 8*

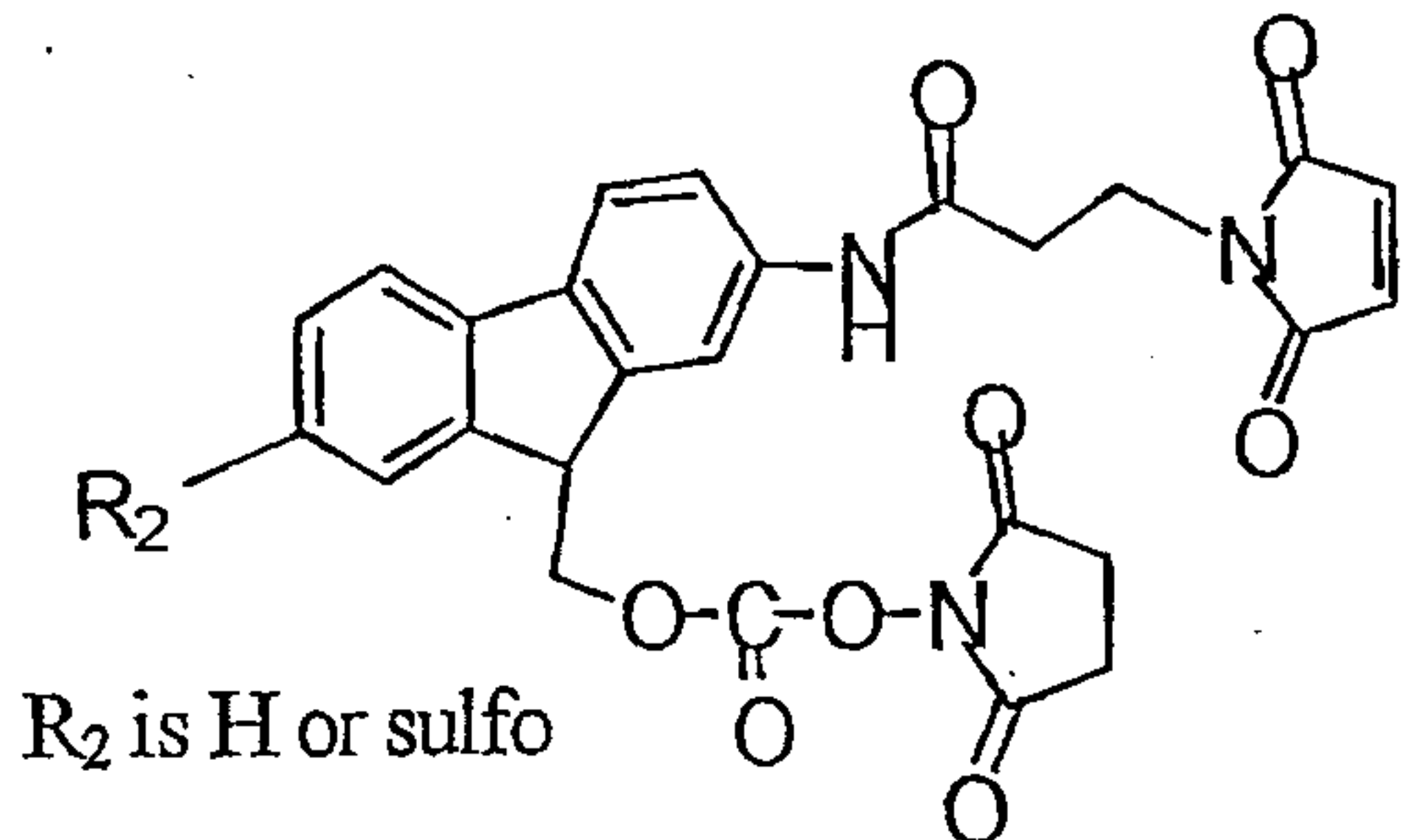
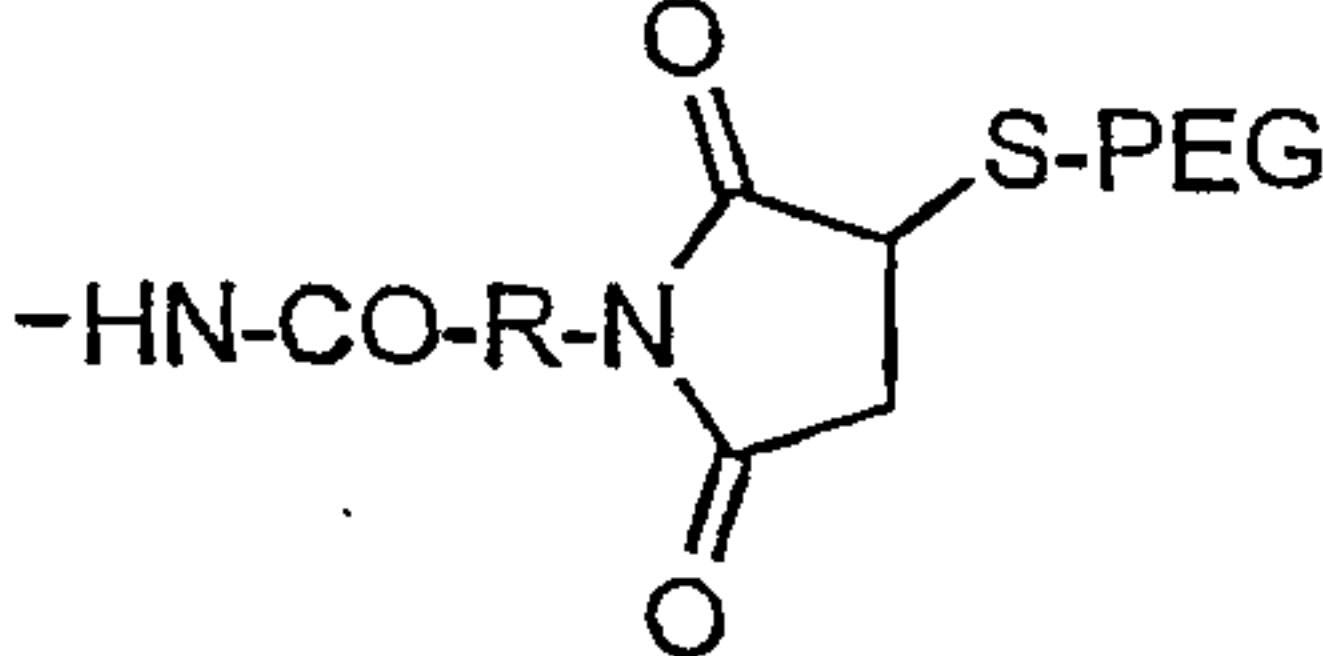
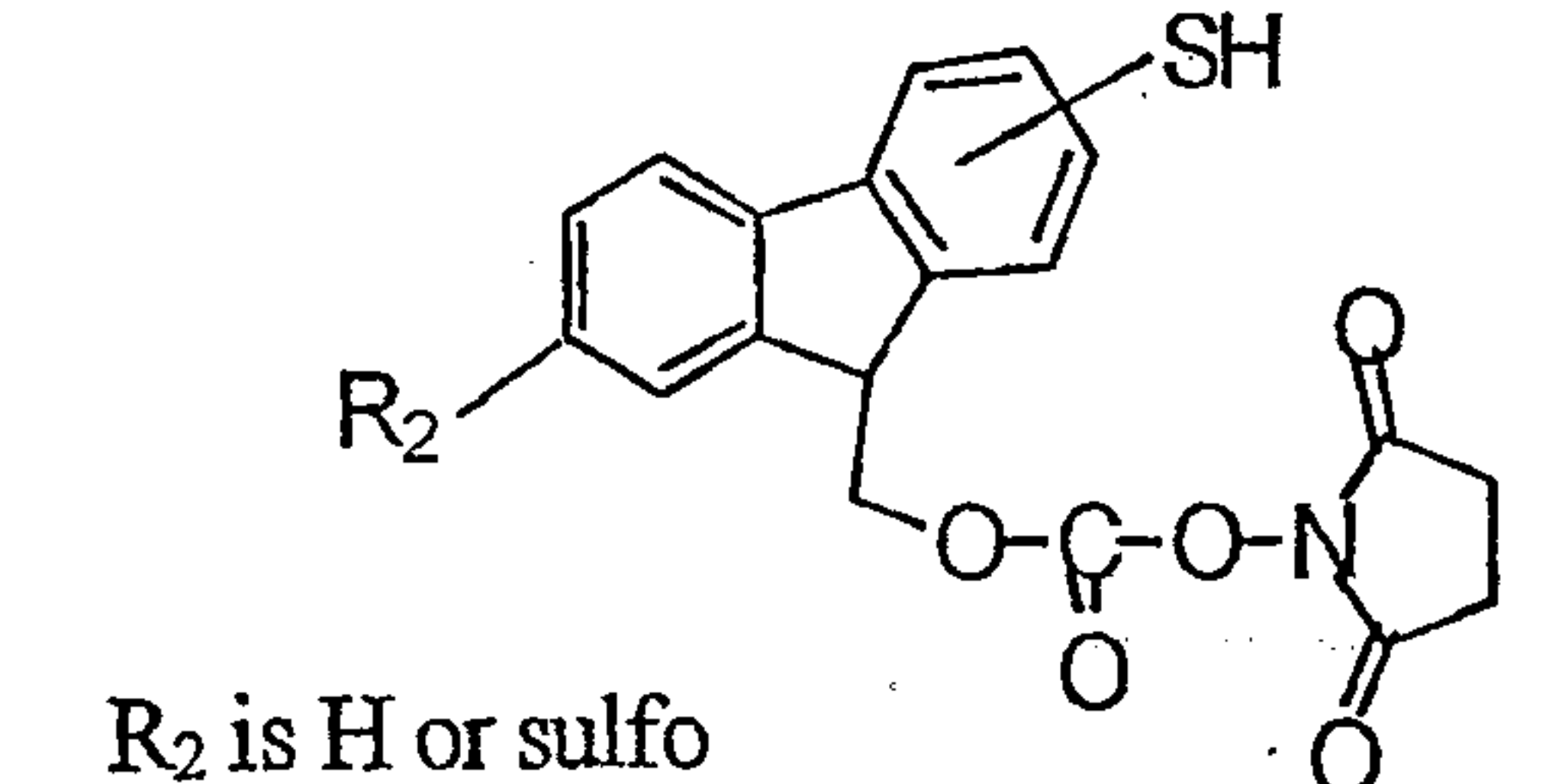
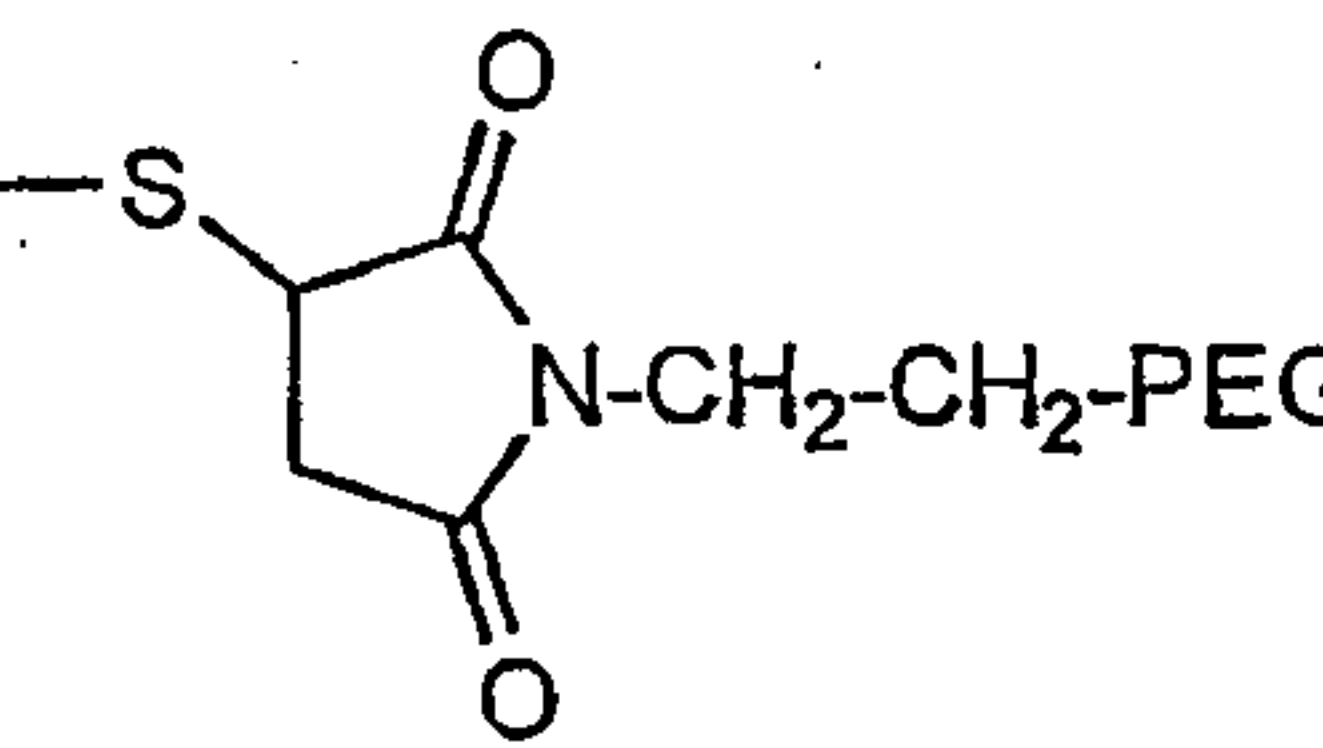
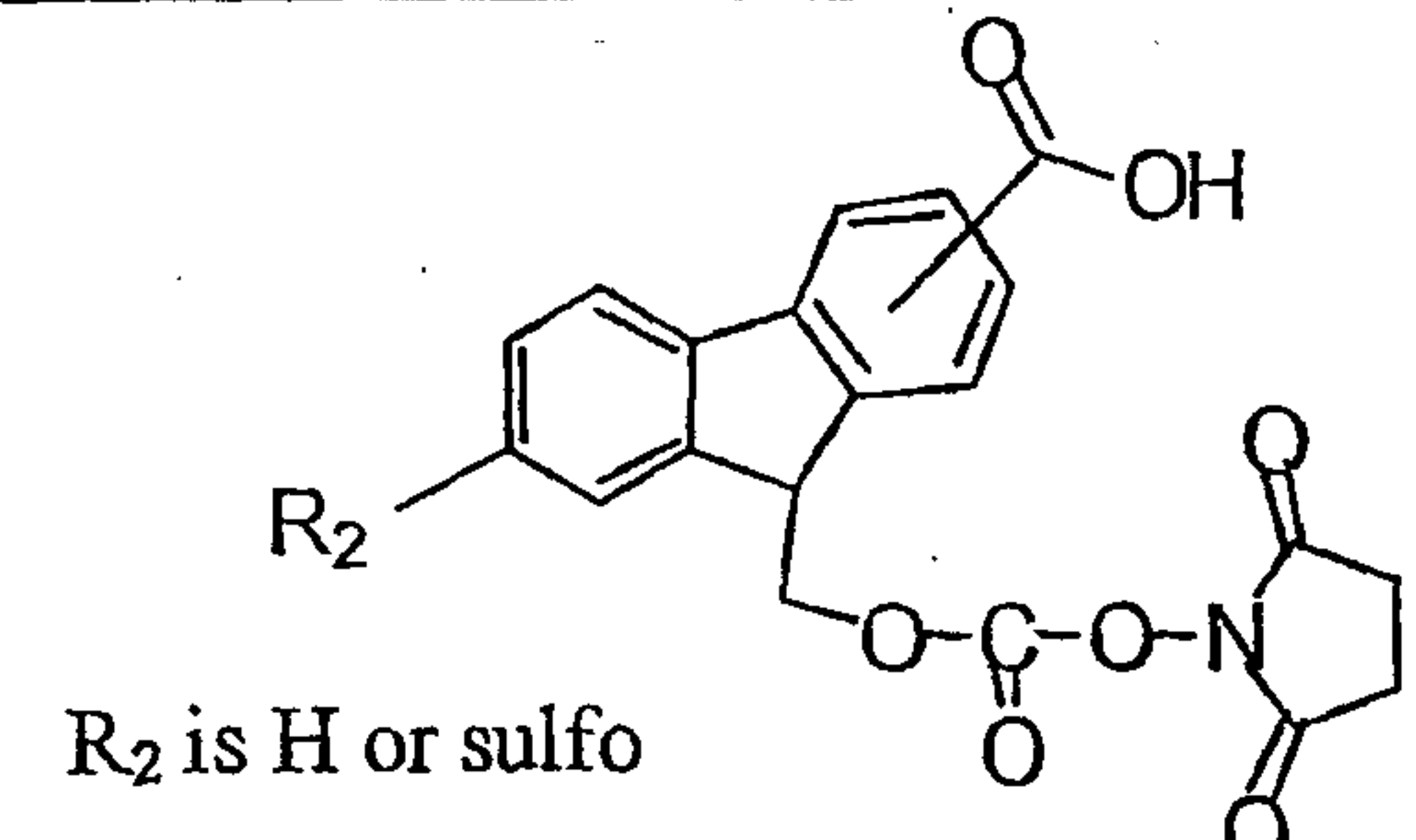
Scheme 6: Procedures for preparation of PEG-FMS-drugs



Scheme 7 (page a)

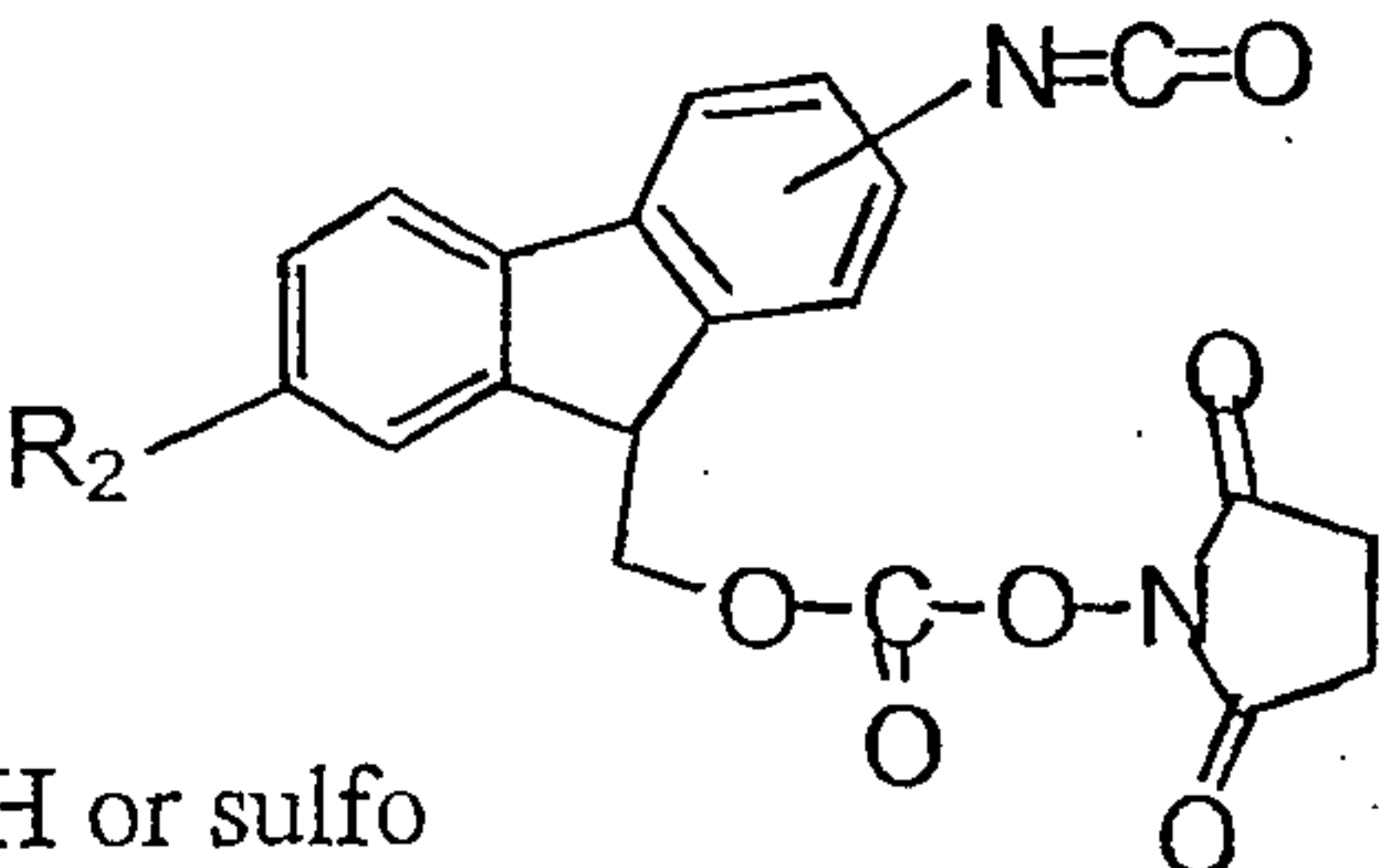
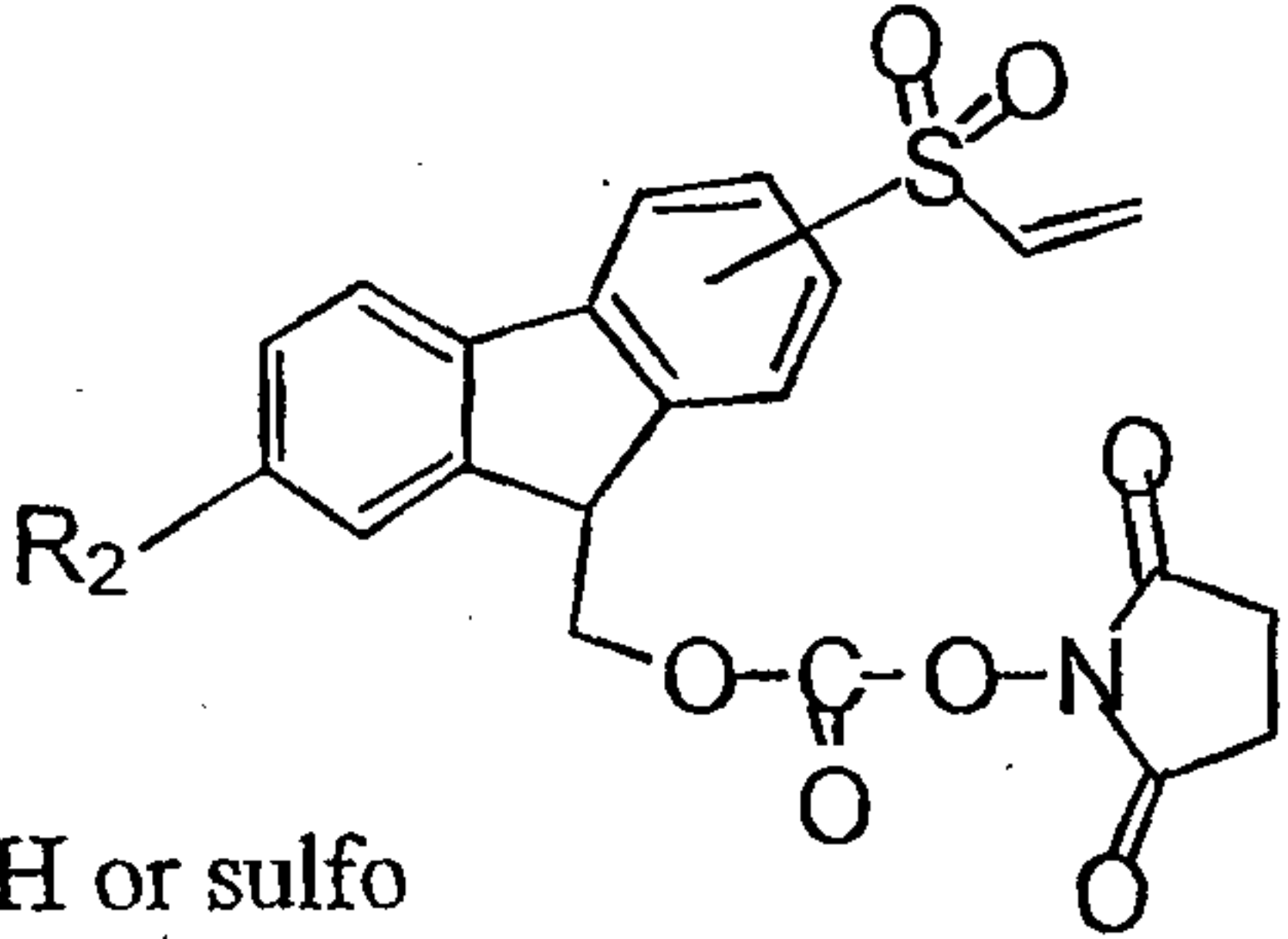
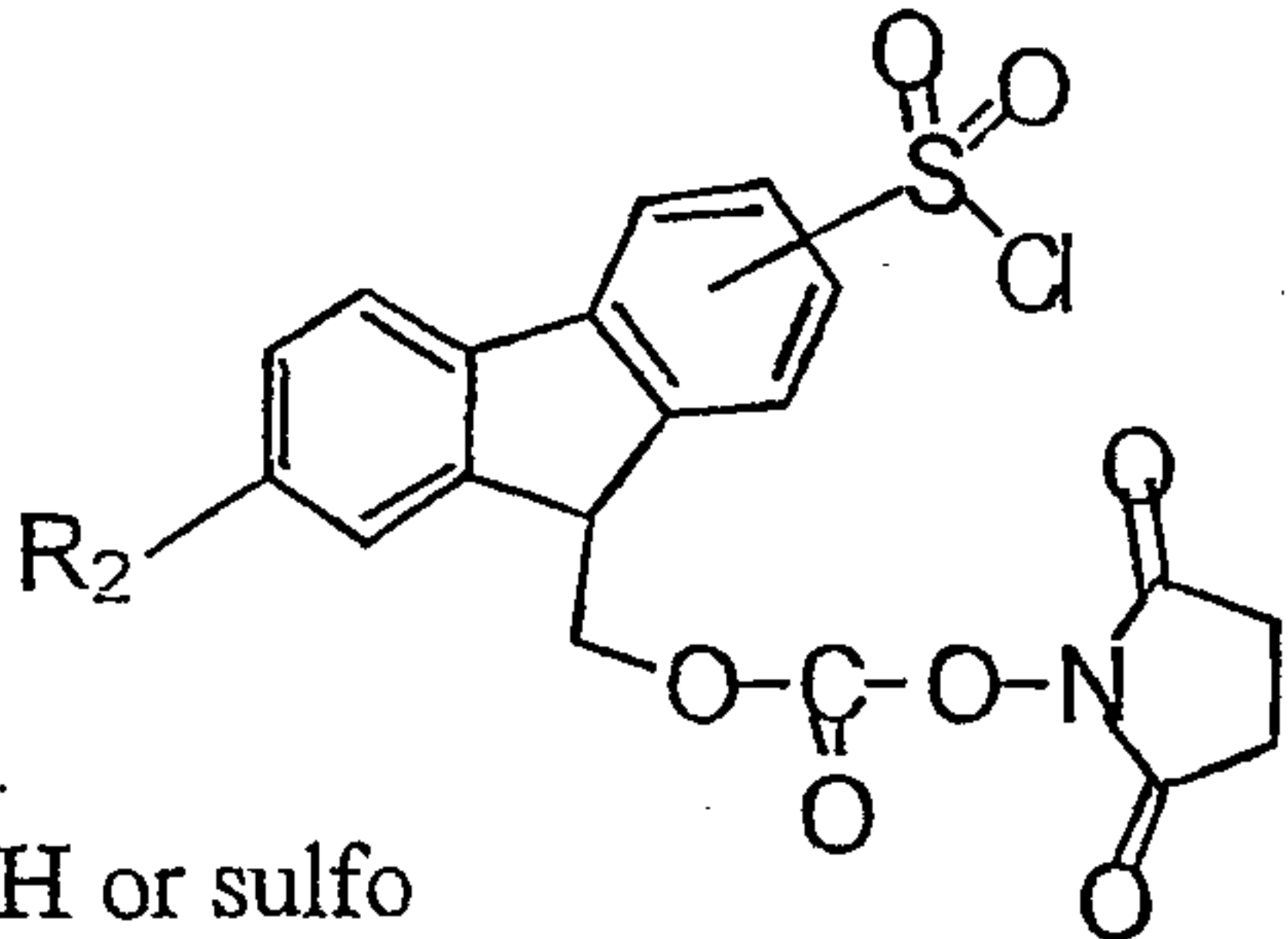
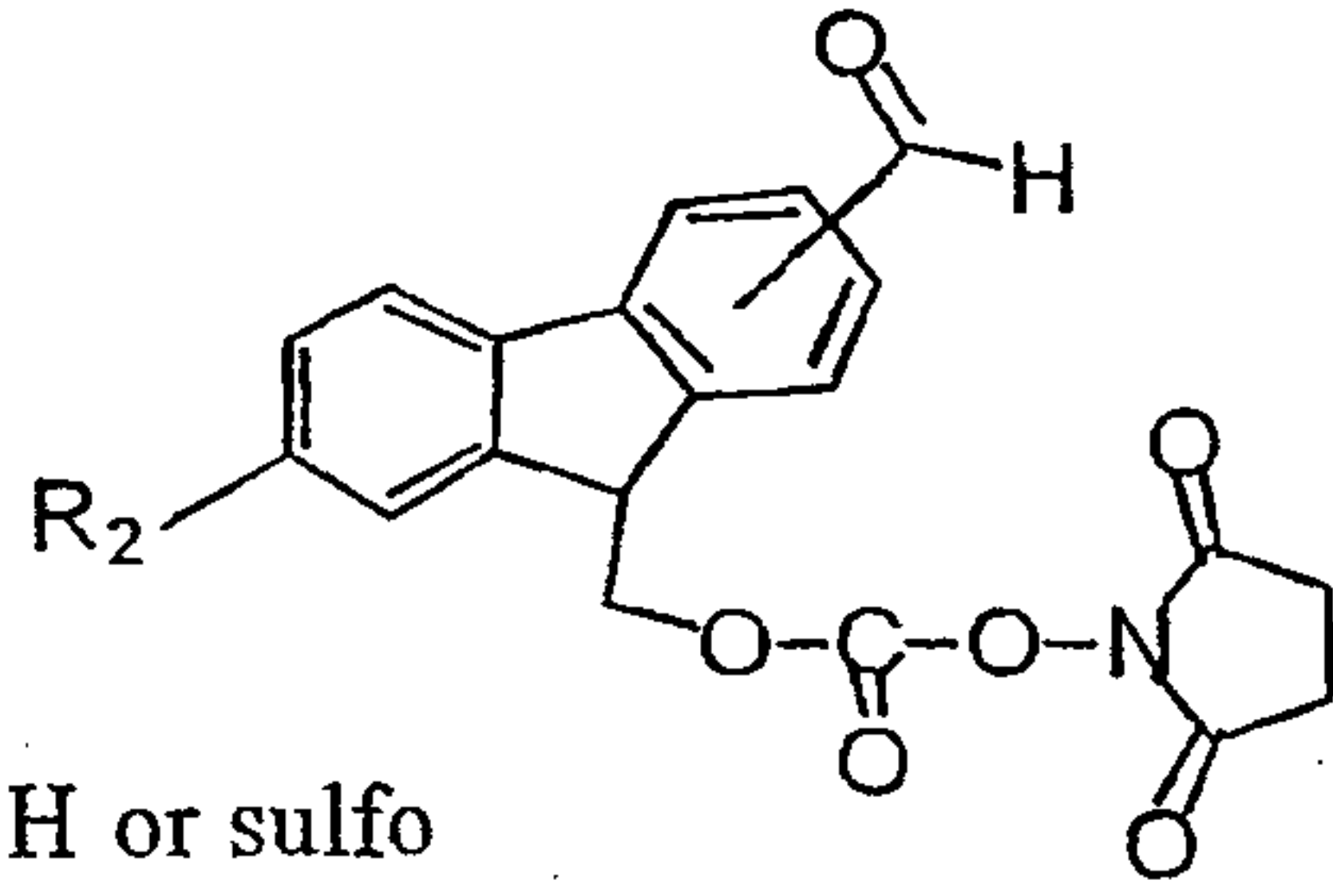
| | Fmoc/FMS derivative | R ₅ -R ₆ -PEG | PEG derivative |
|----|---|--|---|
| 5 |  <p>R₂ is H or sulfo</p> | -NH-CO-O-PEG | PEG-OCO-Cl |
| 10 | | -NH-CO-CH ₂ -NH ₂ -CO-NH-PEG | PEG-NH ₂ |
| | | -NH-CO-PEG | PEG-COOH |
| | | -NH-CH ₂ -PEG | PEG-CHO |
| | | -NH-CO-NH-PEG | PEG-N=C=O |
| 15 | | -NH-CS-NH-PEG | PEG-N=C=S |
| | |  |  |
| 20 | |  |  |
| | |  |  |

Scheme 7 (page b)

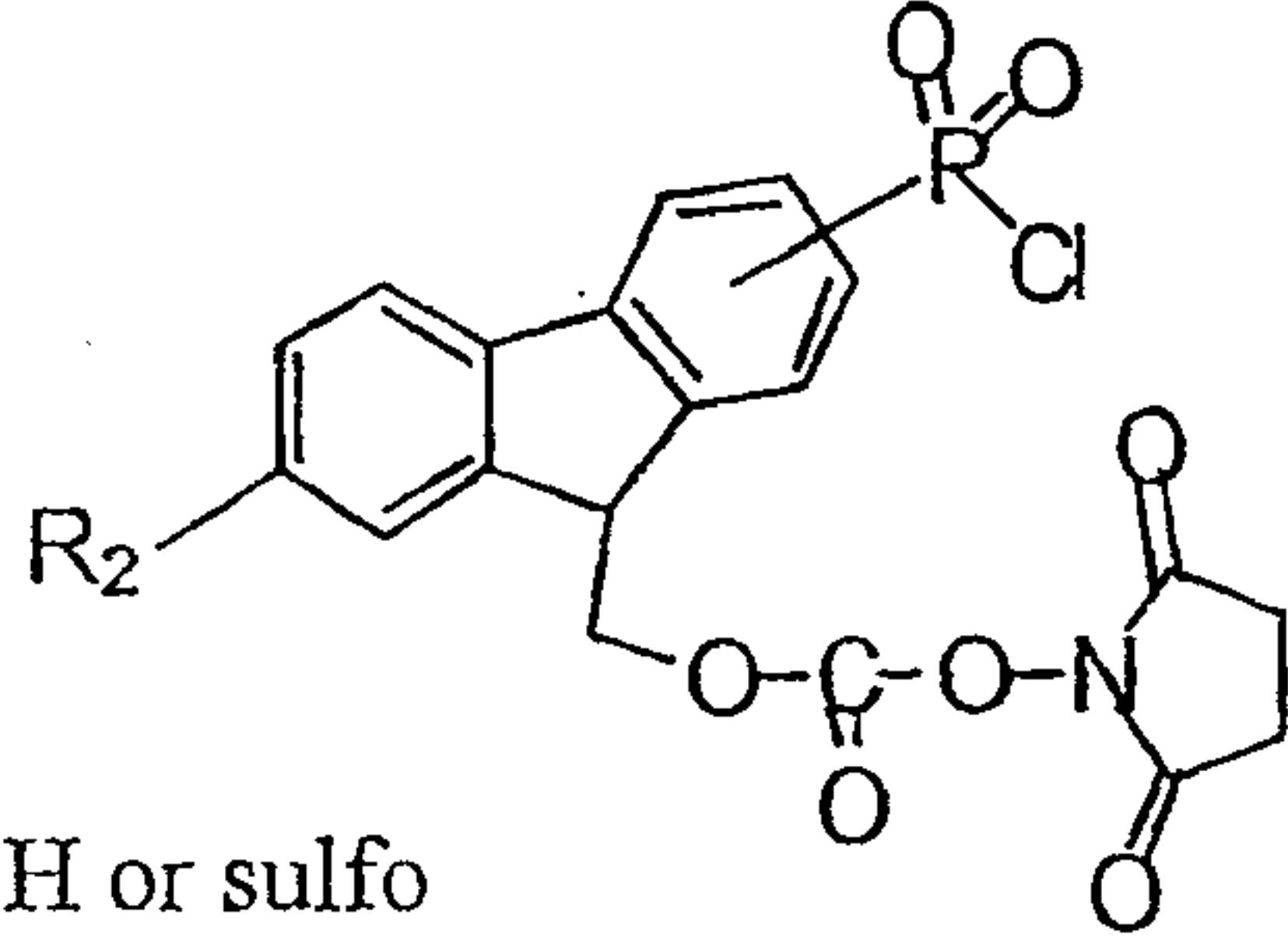
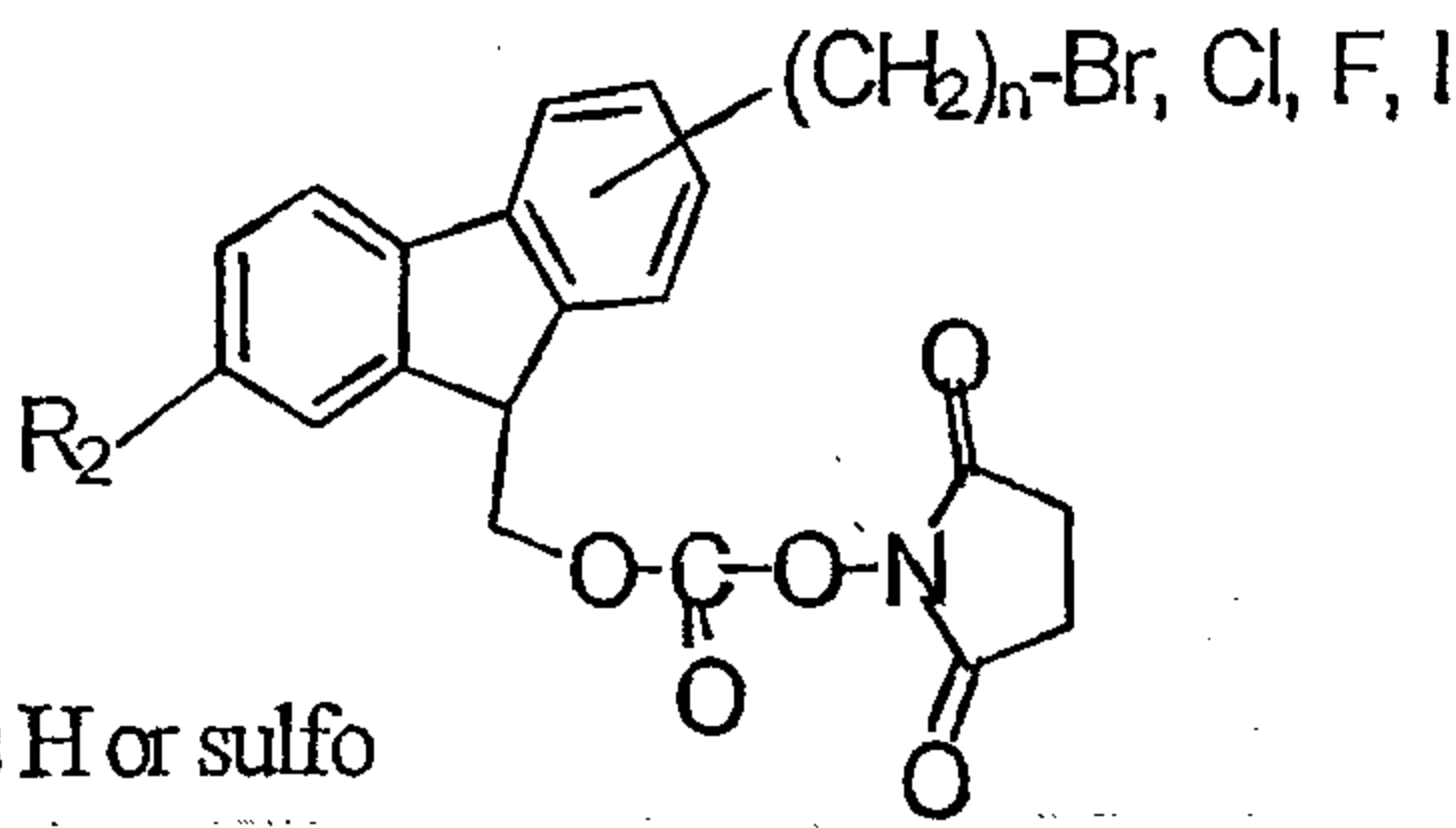
| | Fmoc/FMS derivative | R ₅ -R ₆ -PEG | PEG derivative |
|----|--|---|---|
| 5 |  <p>R₂ is H or sulfo</p> |  <p>-HN-CO-R-N</p> | PEG-SH |
| 10 |  <p>R₂ is H or sulfo</p> |  <p>-S</p> <p>N-CH₂-CH₂-PEG</p> | PEG-CH ₂ -CH ₂ -N |
| 15 |  <p>R₂ is H or sulfo</p> | -CO-O-PEG | PEG-OH |
| | | -CO-NH-PEG | PEG-NH ₂ |

20

Scheme 7 (page c)

| | Fmoc/FMS derivative | R ₅ -R ₆ -PEG | PEG derivative |
|----|--|---|---------------------|
| 5 |  <p>R₂ is H or sulfo</p> | -NH-CO-NH-PEG | PEG-NH ₂ |
| 10 |  <p>R₂ is H or sulfo</p> | -SO ₂ -CH ₂ -CH ₂ -S-PEG | PEG-SH |
| 15 |  <p>R₂ is H or sulfo</p> | -SO ₂ -NH-PEG | PEG-NH ₂ |
| | | -SO ₂ -O-PEG | PEG-OH |
| 20 |  <p>R₂ is H or sulfo</p> | -CH ₂ -NH-PEG | PEG-NH ₂ |

Scheme 7 (page d)

| | Fmoc/FMS derivative | R ₅ -R ₆ -PEG | PEG derivative |
|----|---|---|---------------------|
| 5 |  <p>R₂ is H or sulfo</p> | -PO ₂ -NH-PEG | PEG-NH ₂ |
| | | -PO ₂ -O-PEG | PEG-OH |
| 10 |  <p>R₂ is H or sulfo</p> | -(CH ₂) _{n-1} -CH ₂ -NH-PEG | PEG-NH ₂ |
| 15 | | -(CH ₂) _{n-1} -CH ₂ -S-PEG | PEG-SH |

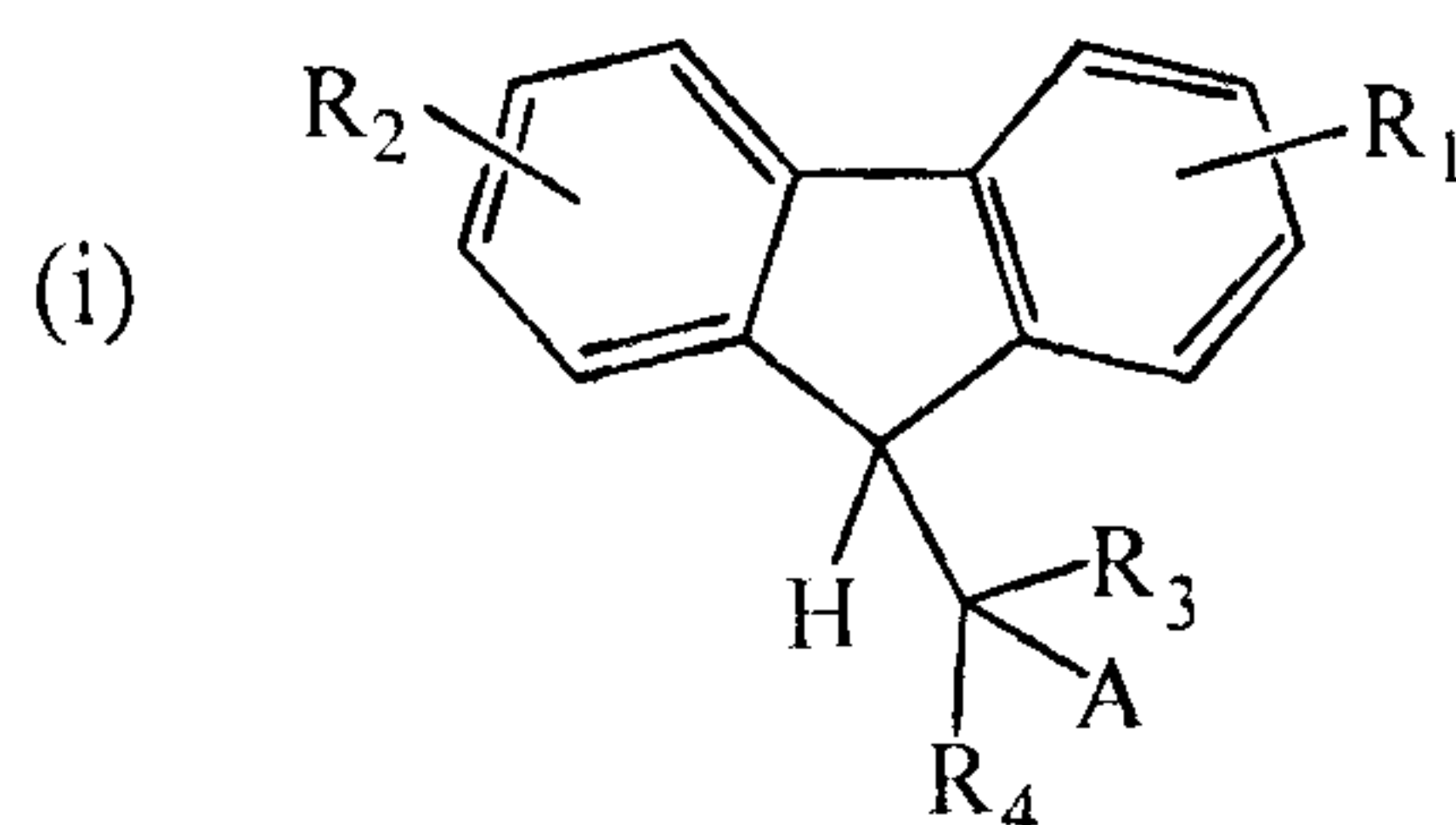
CLAIMS:

1. Use of a conjugate of the formula $(X)_n$ -Y for the preparation of a pharmaceutical composition for treatment of diabetes mellitus or hyperglycemia,

wherein

Y is insulin; and

X is a radical of the formula (i):



wherein

R_1 is a radical containing a linear or branched polyethylene glycol (PEG) moiety;

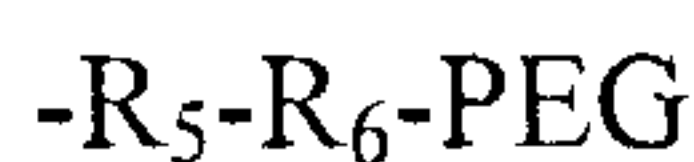
R_2 is selected from hydrogen, (C1-C8)alkyl, (C1-C8) alkoxy, (C1-C8)alkoxyalkyl, (C6-C10)aryl, (C1-C8)alkaryl, (C6-C10)ar(C1-C8)alkyl, halogen, nitro, $-SO_2NHR$, amino, ammonium, carboxyl, PO_3H_2 , or OPO_3H_2 , and R is selected from hydrogen, (C1-C8)alkyl or (C6-C10)aryl;

R_3 and R_4 , the same or different, are each selected from hydrogen, (C1-C8)alkyl or (C6-C10)aryl; and

A is OCO- linked to an amino group of the insulin; and

n is an integer of at least one.

2. The use of claim 1, wherein R_2 , R_3 and R_4 each is hydrogen.
3. The use of claim 1, wherein said insulin is human insulin.
4. The use of claim 1, wherein R_1 is a radical of the formula:



wherein

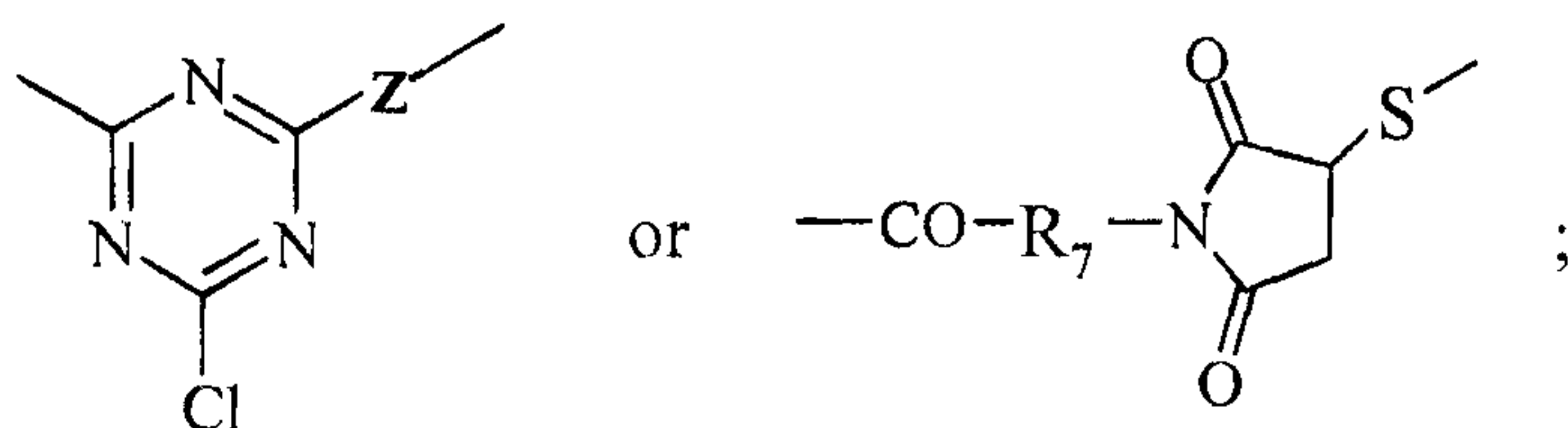
R_5 is $-NH-$, $-S-$, $-CO-$, $-COO-$, $-CH_2-$, $-SO_2-$, $-SO_3-$, $-PO_2-$, or $-PO_3-$, and

R_6 is a bond or a radical through which the PEG moiety is covalently linked to R_5 .

5. The use of claim 4, wherein:

R_5 is -NH-;

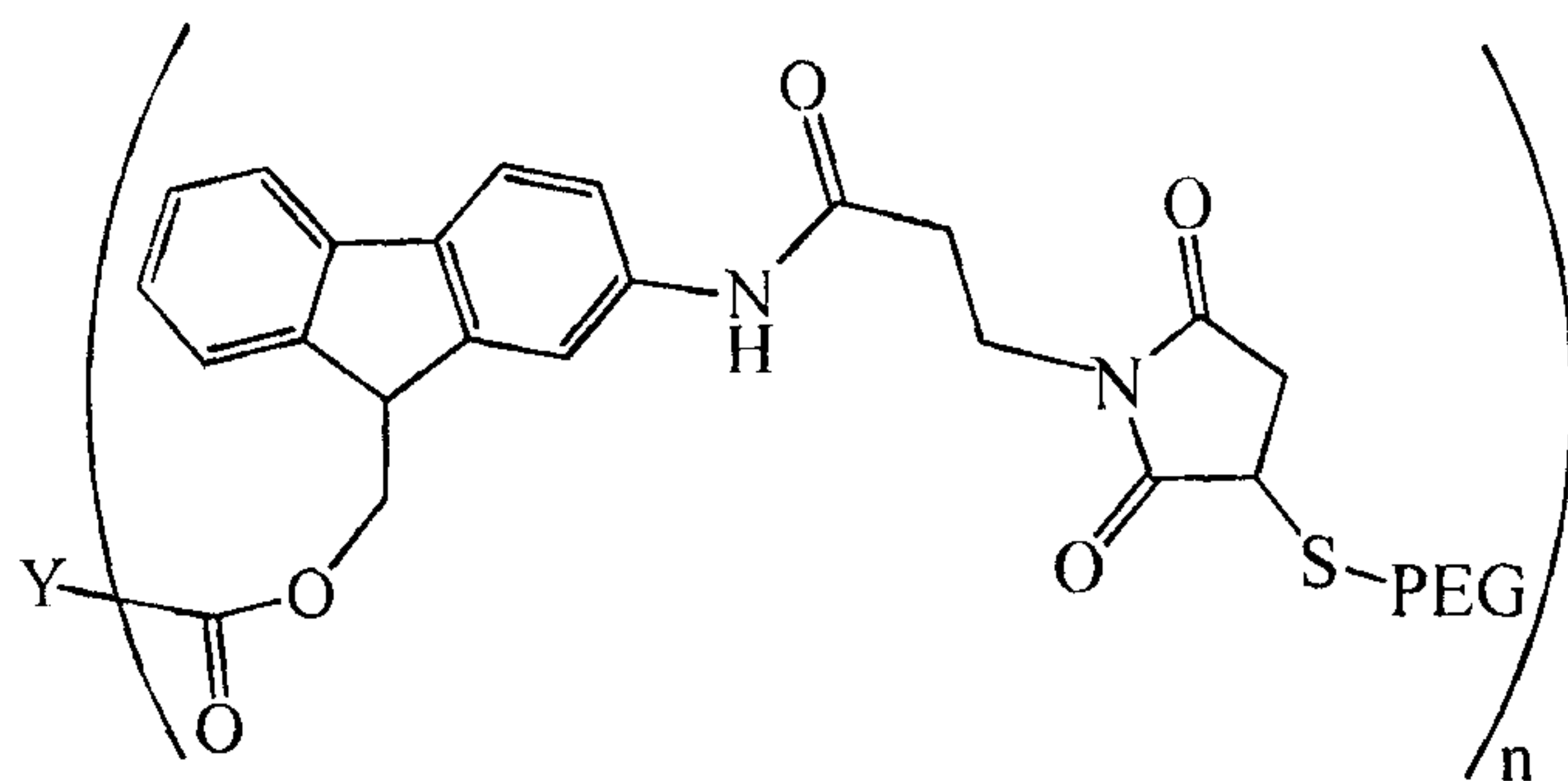
R_6 is -CO-, -COO-, -CH₂-, -CH(CH₃)-, CO-NH-, -CS-NH-, -CO-CH₂-NH-CO-, -CO-CH(CH₃)-NH-CO-, -CO-CH₂-NH-CO-NH,



Z is O, S or NH that is linked to the PEG moiety; and

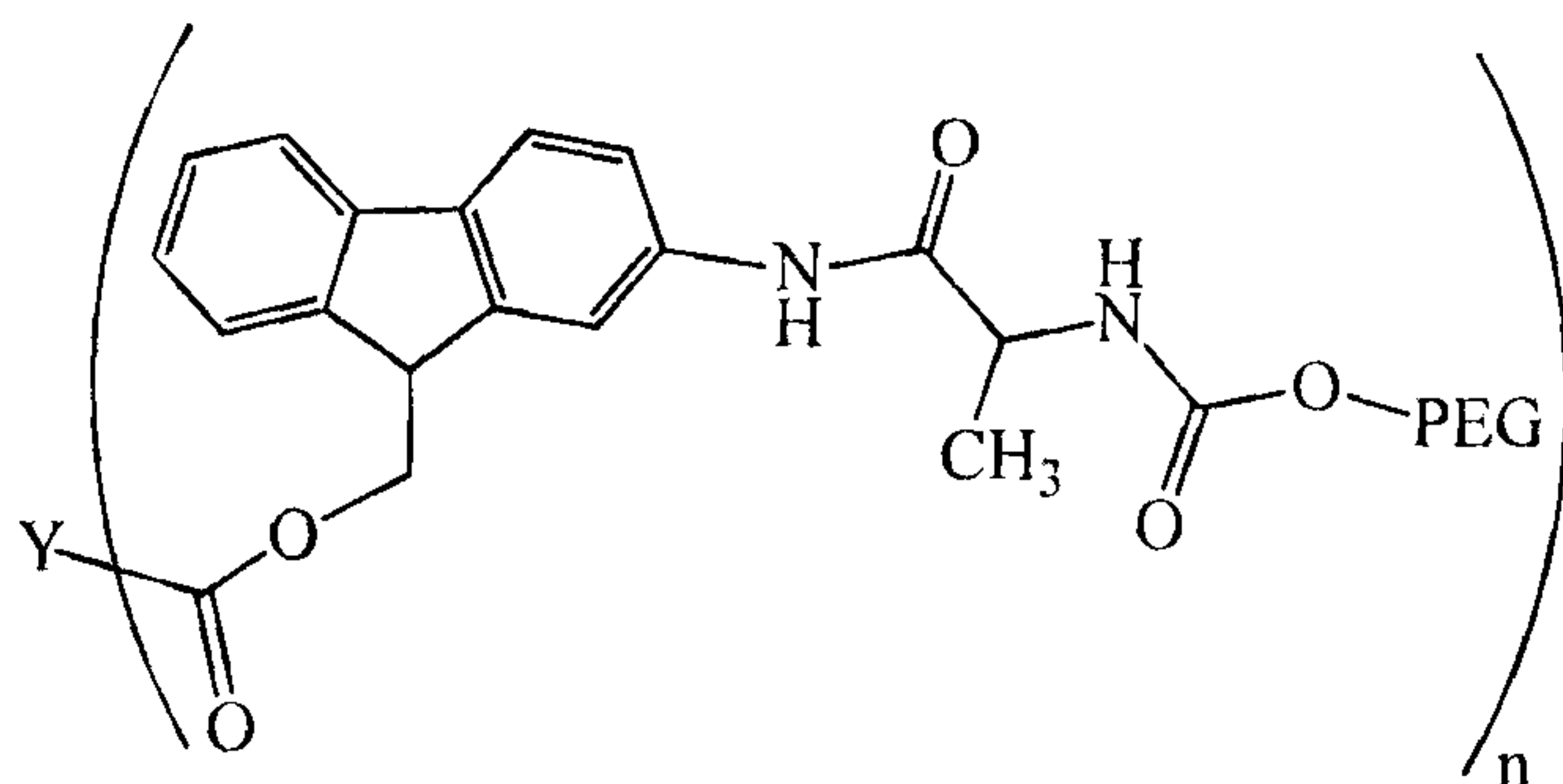
R_7 is selected from C1-C18 straight or branched alkylene, phenylene, an oxyalkylene radical having 3-18 carbon atoms in the backbone, a residue of a peptide containing 2-10 amino acid residues, or a residue of a saccharide containing 1-10 monosaccharide residues.

6. The use of claim 5, wherein said conjugate has the formula:



wherein PEG is a linear or branched PEG moiety of molecular weight in the range of 200 to 200,000 Da.

7. The use of claim 5, wherein said conjugate has the formula:



wherein PEG is a linear or branched PEG moiety of molecular weight in the range of 200 to 200,000 Da.

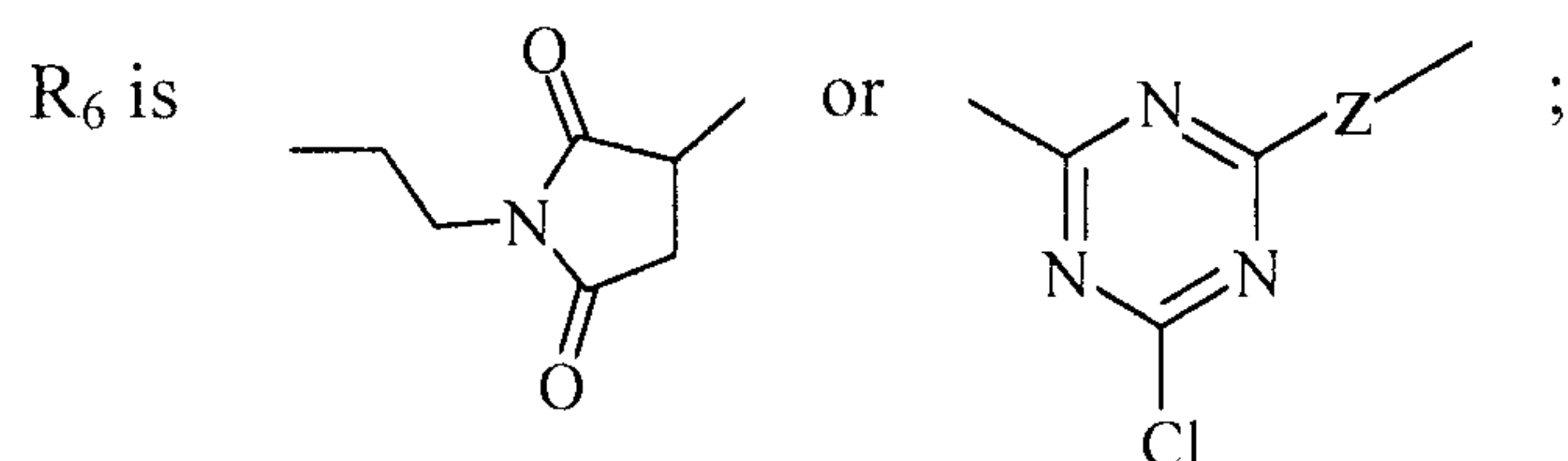
8. The use of claim 6 or 7, wherein n is an integer from 1 to 3.

9. The use of claim 8, wherein the PEG moiety has a molecular weight in the range of 5000 to 40000 Da.

10. The use of claim 9, wherein the PEG moiety is a branched molecule of 40000 Da or 5000 Da.

11. The use of claim 4, wherein:

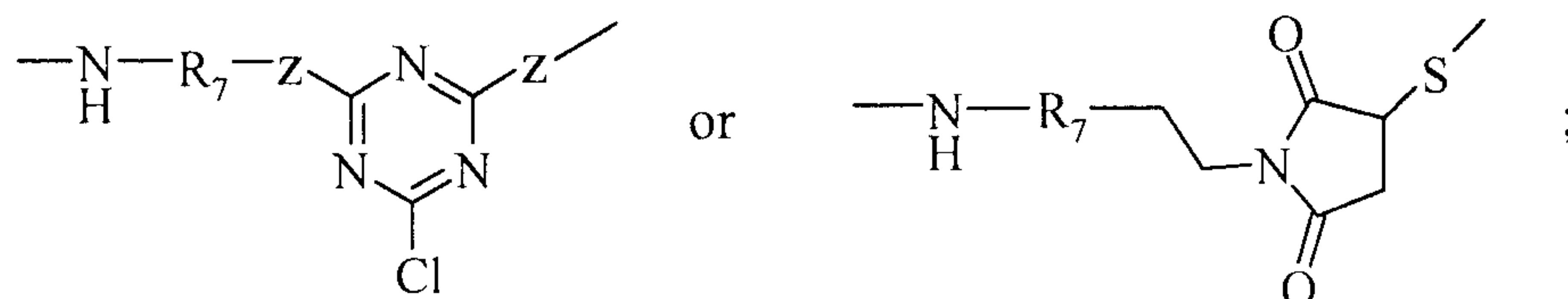
(i) R_5 is -S-;



Z is O, S or NH;

(ii) R_5 is -CO; and

R_6 is O-; -NH-; -NH- R_7 -COO-; -NH- R_7 -NH; -NH- R_7 -CO-NH;



Z is O, S or NH; and

R_7 is selected from C1-C18 straight or branched alkylene, phenylene, an oxyalkylene radical having 3-18 carbon atoms in the backbone, a residue of a peptide containing 2-10 amino acid residues, or a residue of a saccharide containing 1-10 monosaccharide residues;

(iii) R_5 is -CH₂-; and

R_6 is -(CH₂)_n-S- or -(CH₂)_n-NH-, wherein n is 0 to 18;

(iv) R_5 is -SO₂-; and

R_6 is O; -NH- or -CH₂-CH₂-S; or

(v) R_5 is -PO₂-; and

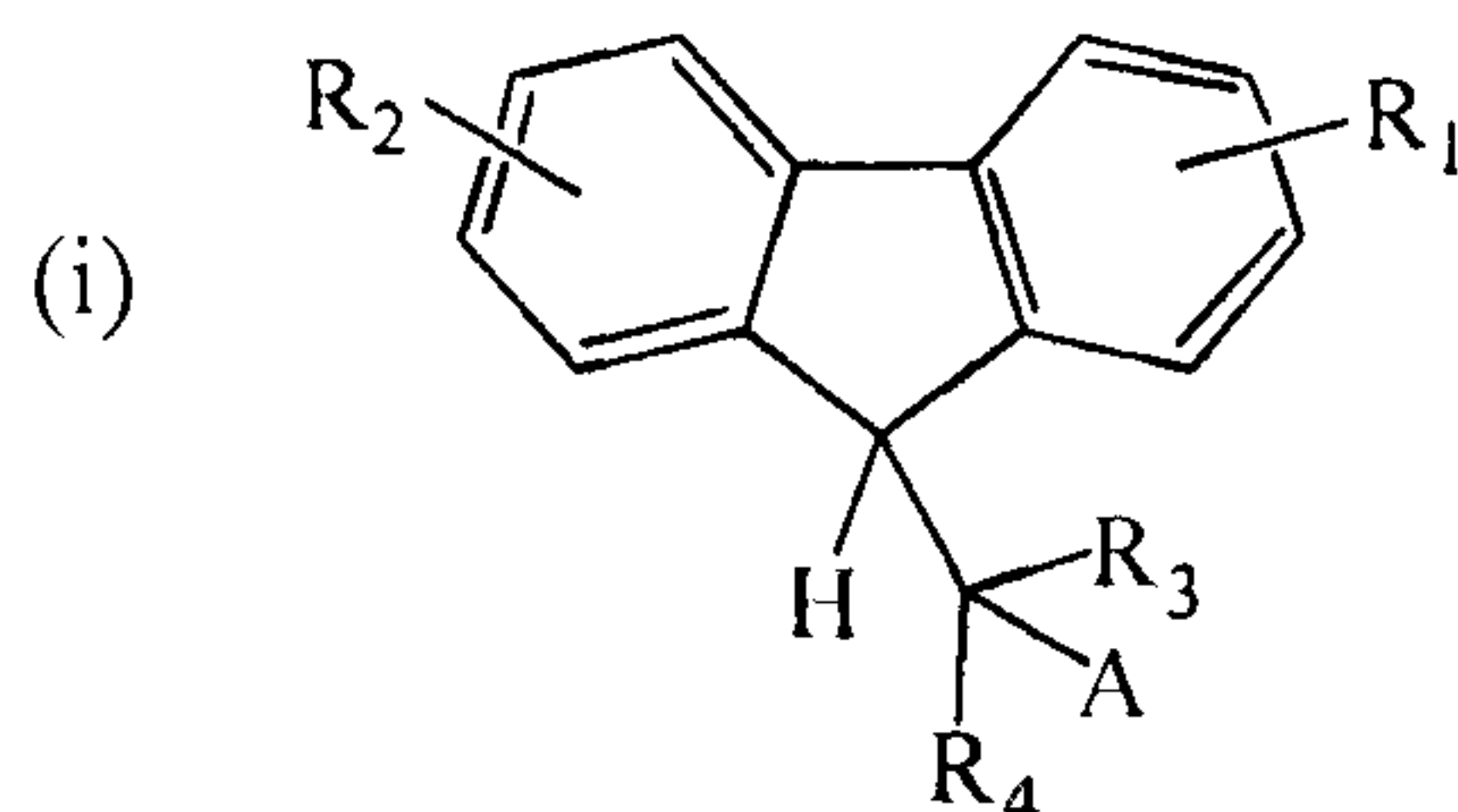
R_6 is -O- or -NH-.

12. Use of a conjugate of the formula $(X)_n$ -Y for the preparation of a pharmaceutical composition for treatment of insulin-dependent diabetes mellitus, non-insulin-dependent diabetes mellitus, or gestational diabetes mellitus, or for prevention of hyperglycemia,

wherein

Y is an exendin, exendin analogue, or exendin agonist; and

X is a radical of the formula (i):



wherein

R_1 is a radical containing a linear or branched polyethylene glycol (PEG) moiety;

R_2 is selected from hydrogen, (C1-C8)alkyl, (C1-C8)alkoxy, (C1-C8)alkoxyalkyl, (C6-C10)aryl, (C1-C8)alkaryl, (C6-C10)ar(C1-C8)alkyl, halogen, nitro, -SO₂NHR, amino, ammonium, carboxyl, PO₃H₂, or OPO₃H₂, and R is selected from hydrogen, (C1-C8)alkyl or (C6-C10)aryl;

R_3 and R_4 , the same or different, are each selected from hydrogen, (C1-C8)alkyl or (C6-C10)aryl; and

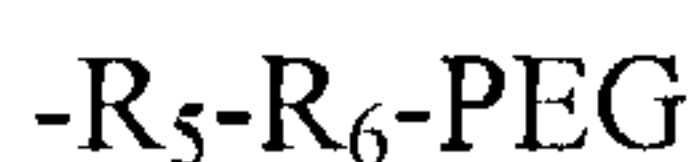
A is OCO- linked to an amino group of the insulin; and

n is an integer of at least one.

13. The use of claim 12, wherein R_2 , R_3 and R_4 each is hydrogen.

14. The use of claim 12, wherein said exendin is exendin-4 [SEQ ID NO: 1] or exendin-3 [SEQ ID NO: 2], and said exendin agonist is [SEQ ID NO:3], [SEQ ID NO:4], [SEQ ID NO:5], [SEQ ID NO:6], [SEQ ID NO:7], [SEQ ID NO:8], [SEQ ID NO:9] or [SEQ ID NO:10].

15. The use of claim 12, wherein R_1 is a radical of the formula:



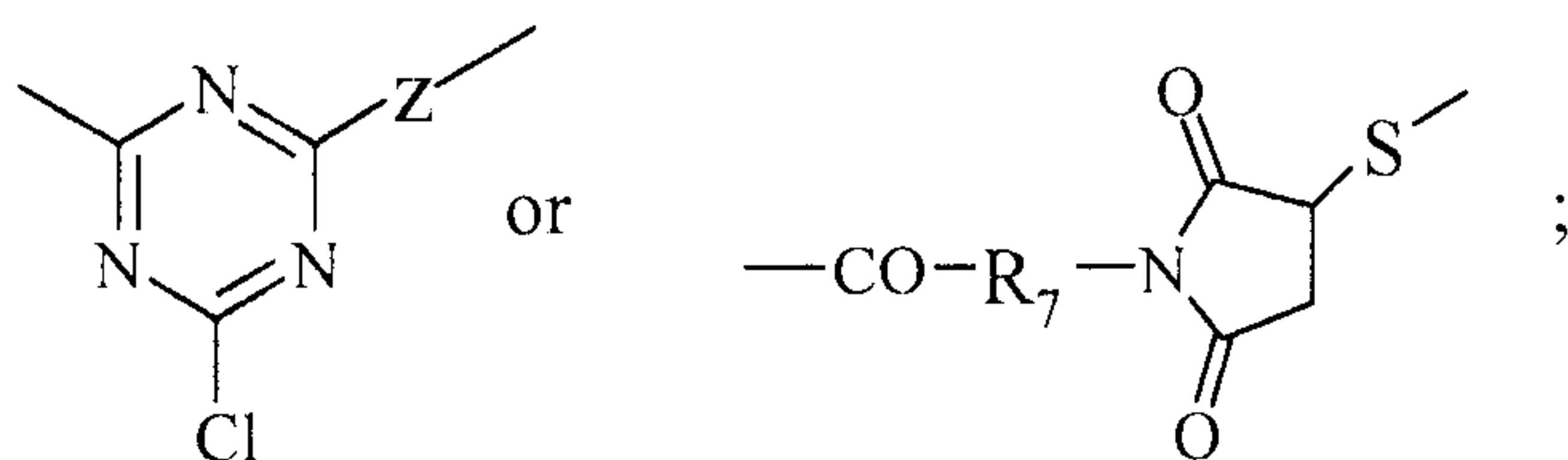
wherein

R_5 is -NH-, -S-, -CO-, -COO-, -CH₂-, -SO₂-, -SO₃-, -PO₂-, or -PO₃-, and
 R_6 is a bond or a radical through which the PEG moiety is covalently linked to R_5 .

16. The use of claim 15, wherein:

R_5 is -NH-;

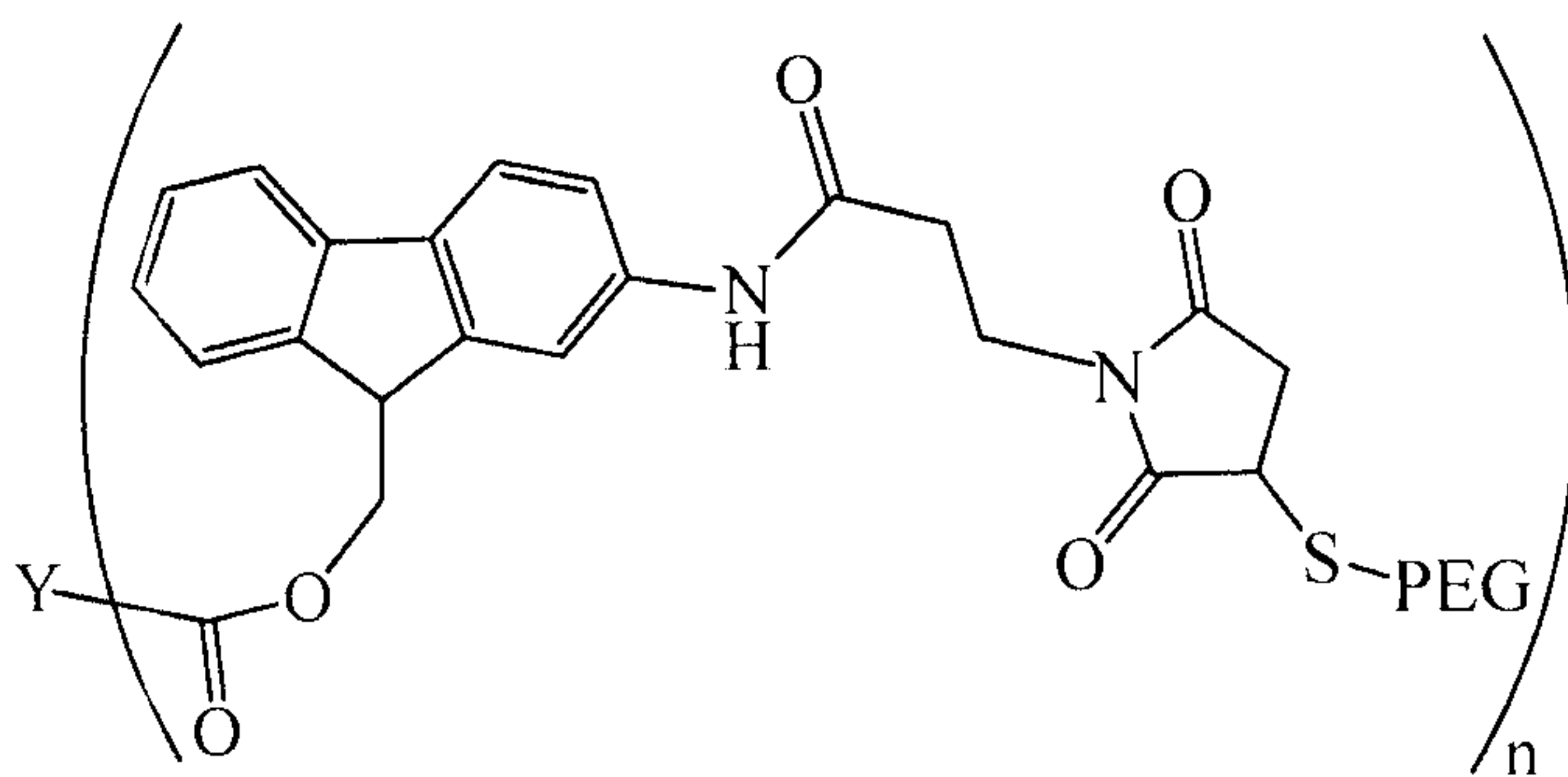
R_6 is -CO-, -COO-, -CH₂-, -CH(CH₃)-, CO-NH-, -CS-NH-, -CO-CH₂-NH-CO-, -CO-CH(CH₃)-NH-CO-, -CO-CH₂-NH-CO-NH,



Z is O, S or NH that is linked to the PEG moiety; and

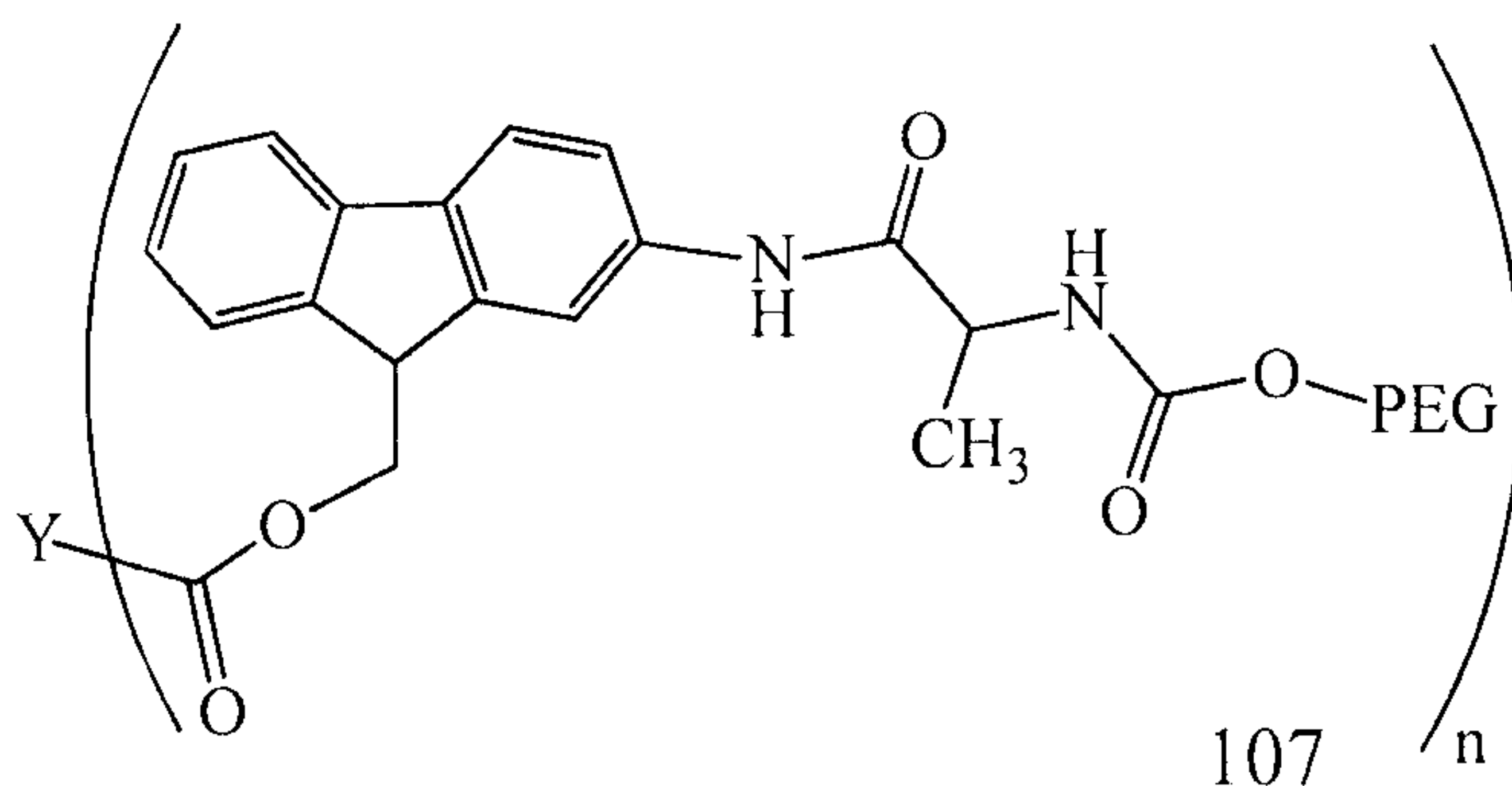
R_7 is selected from C1-C18 straight or branched alkylene, phenylene, an oxyalkylene radical having 3-18 carbon atoms in the backbone, a residue of a peptide containing 2-10 amino acid residues, or residue of a saccharide containing 1-10 monosaccharide residues.

17. The use of claim 16, wherein said conjugate has the formula:



wherein PEG is a linear or branched PEG moiety of molecular weight in the range of 200 to 200,000 Da.

18. The use of claim 16, wherein said conjugate has the formula:



wherein PEG is a linear or branched PEG moiety of molecular weight in the range of 200 to 200,000 Da.

19. The use of claim 17 or 18, wherein n is an integer from 1 to 3.

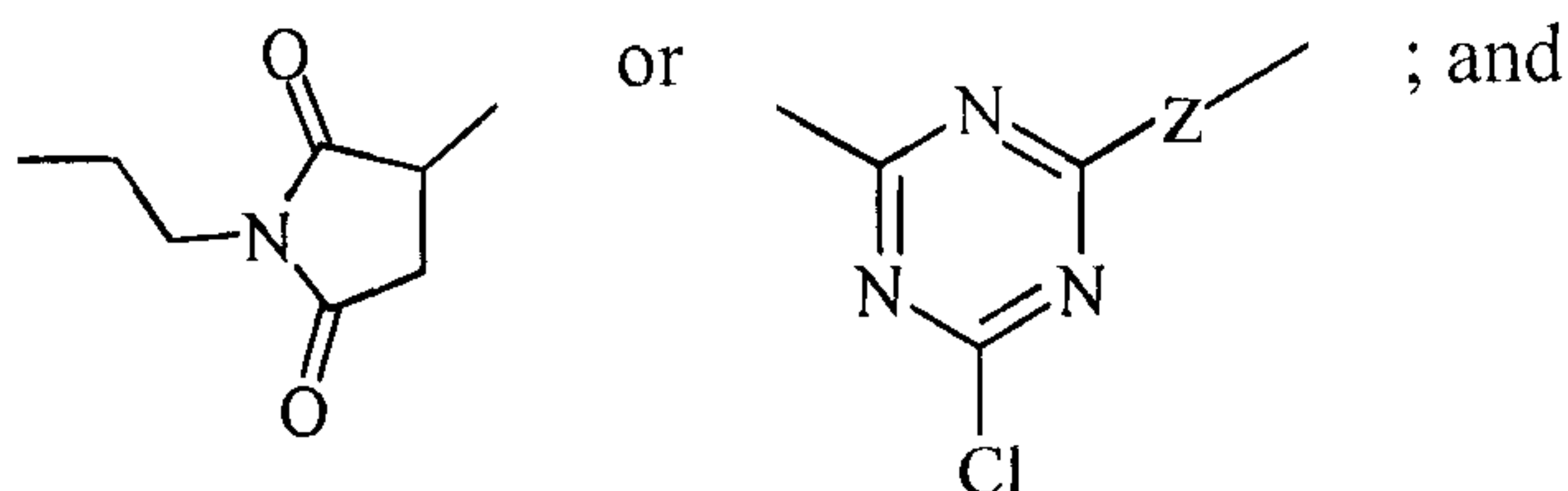
20. The use of claim 19, wherein the PEG moiety has a molecular weight in the range of 5000 to 40000 Da.

21. The use of claim 20, wherein the PEG moiety is a branched molecule of 40000 Da or 5000 Da.

22. The use of claim 15, wherein:

(i) R_5 is -S-;

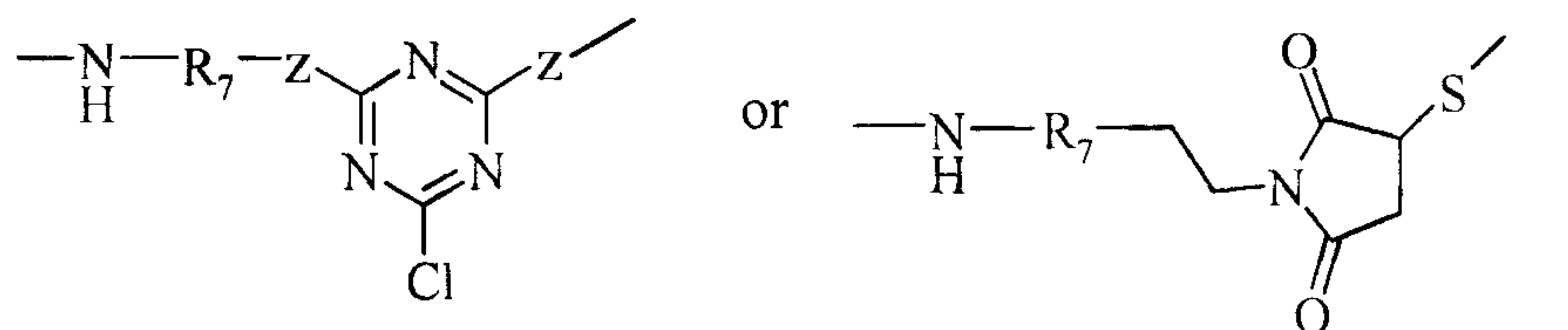
R_6 is



Z is O, S or NH;

(ii) R_5 is -CO; and

R_6 is O-; -NH-; -NH- R_7 -COO-; -NH- R_7 -NH; -NH- R_7 -CO-NH;



Z is O, S or NH; and

R_7 is selected from C1-C18 straight or branched alkylene, phenylene, an oxyalkylene radical having 3-18 carbon atoms in the backbone, a residue of a peptide containing 2-10 amino acid residues, or a residue of a saccharide containing 1-10 monosaccharide residues;

(iii) R_5 is -CH₂-; and

R_6 is -(CH₂)_n-S- or -(CH₂)_n-NH-, wherein n is 0 to 18;

(iv) R_5 is -SO₂-; and

R_6 is O; -NH- or -CH₂-CH₂-S; or

- (v) R_5 is $-\text{PO}_2-$; and
 R_6 is $-\text{O}-$ or $-\text{NH}-$.

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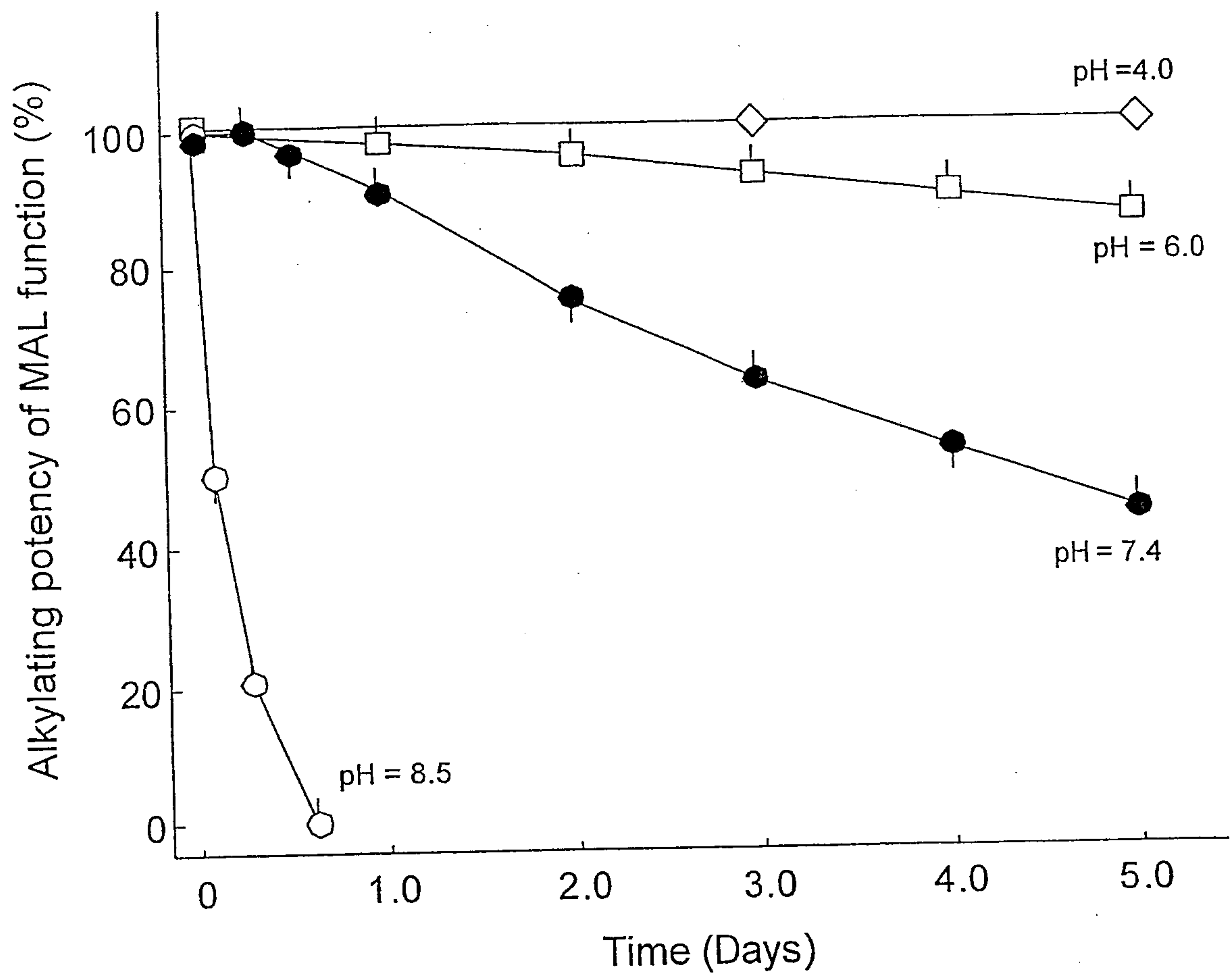
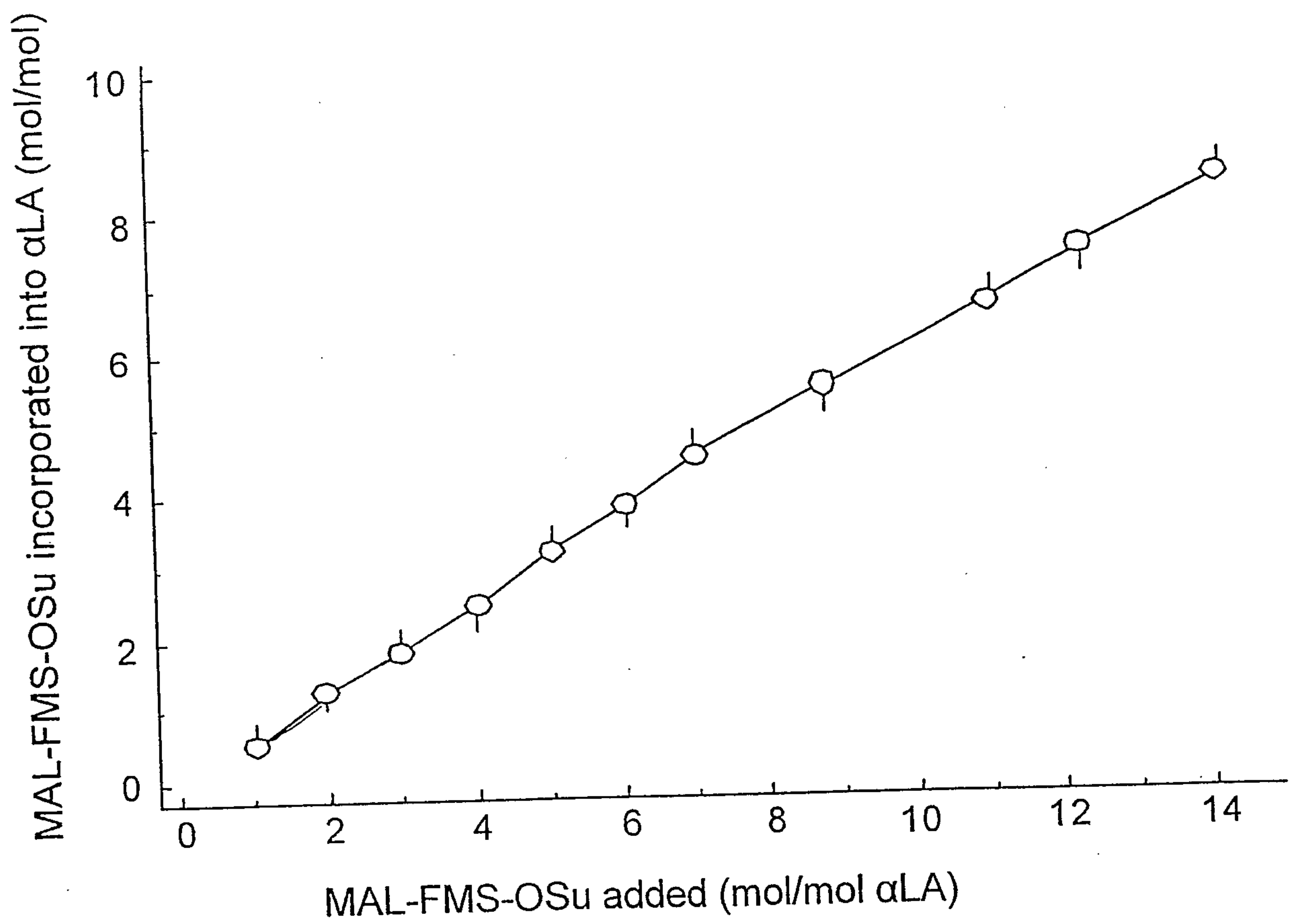


Fig. 1

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**Fig. 2**

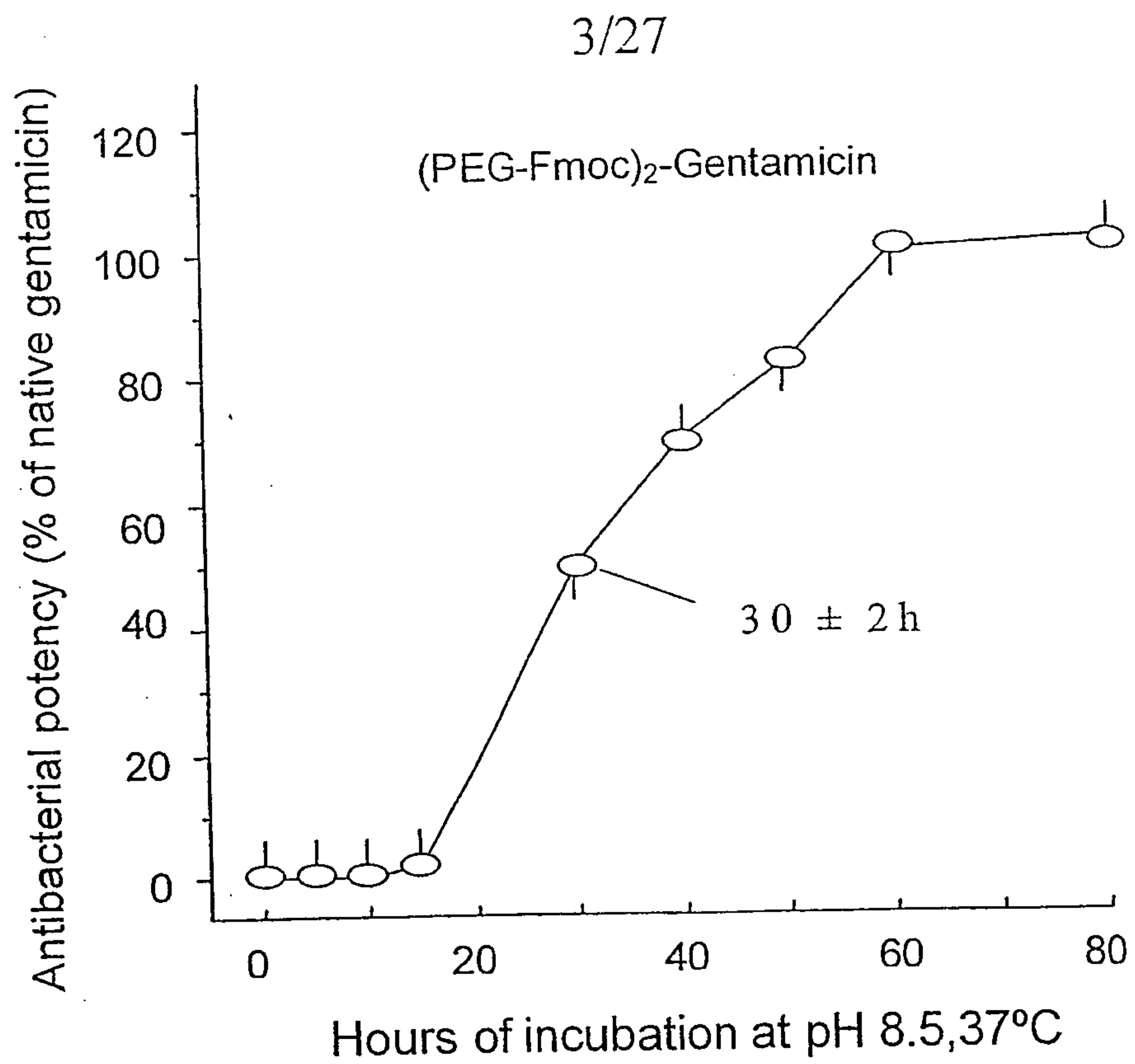


Fig. 3A

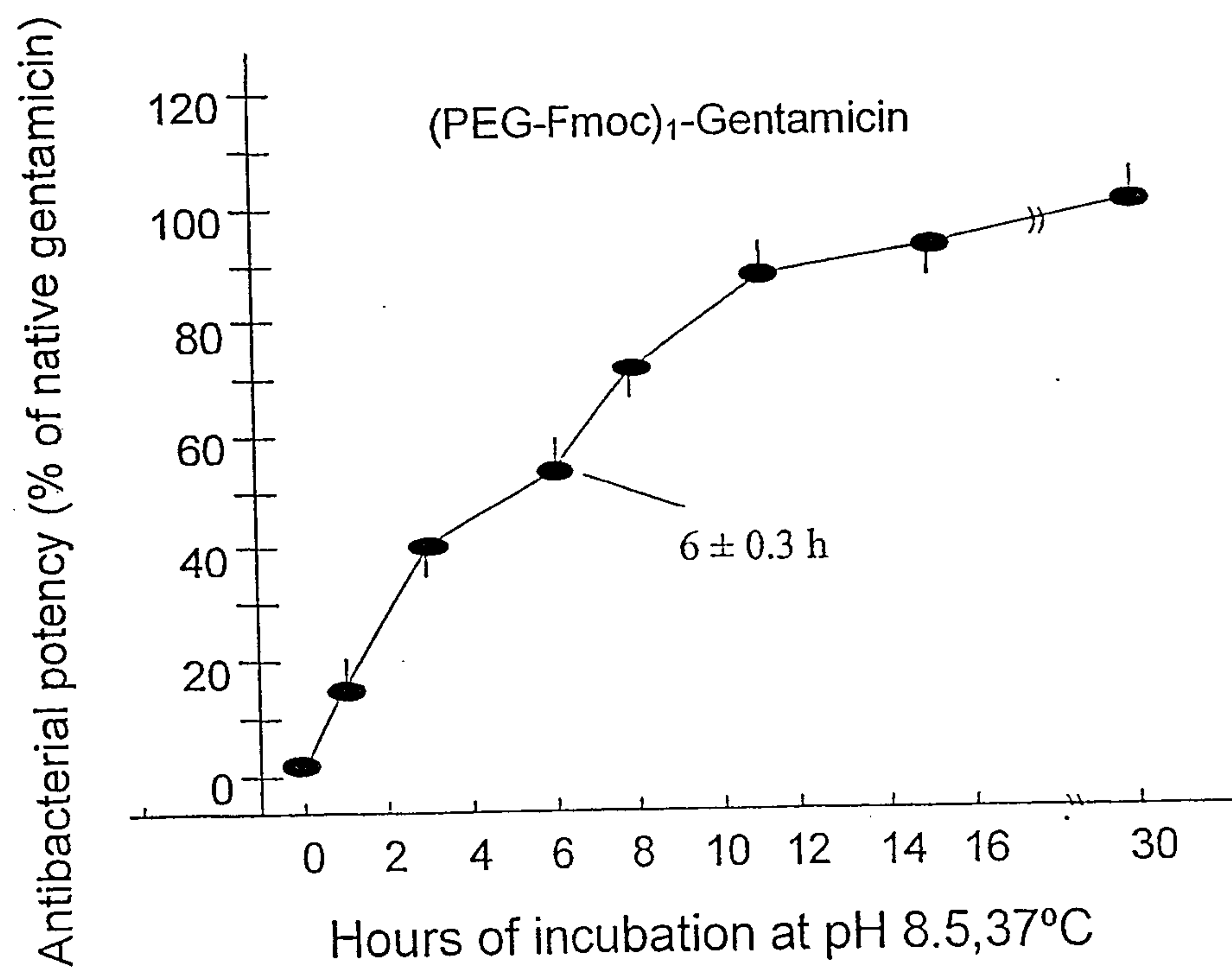


Fig. 3B

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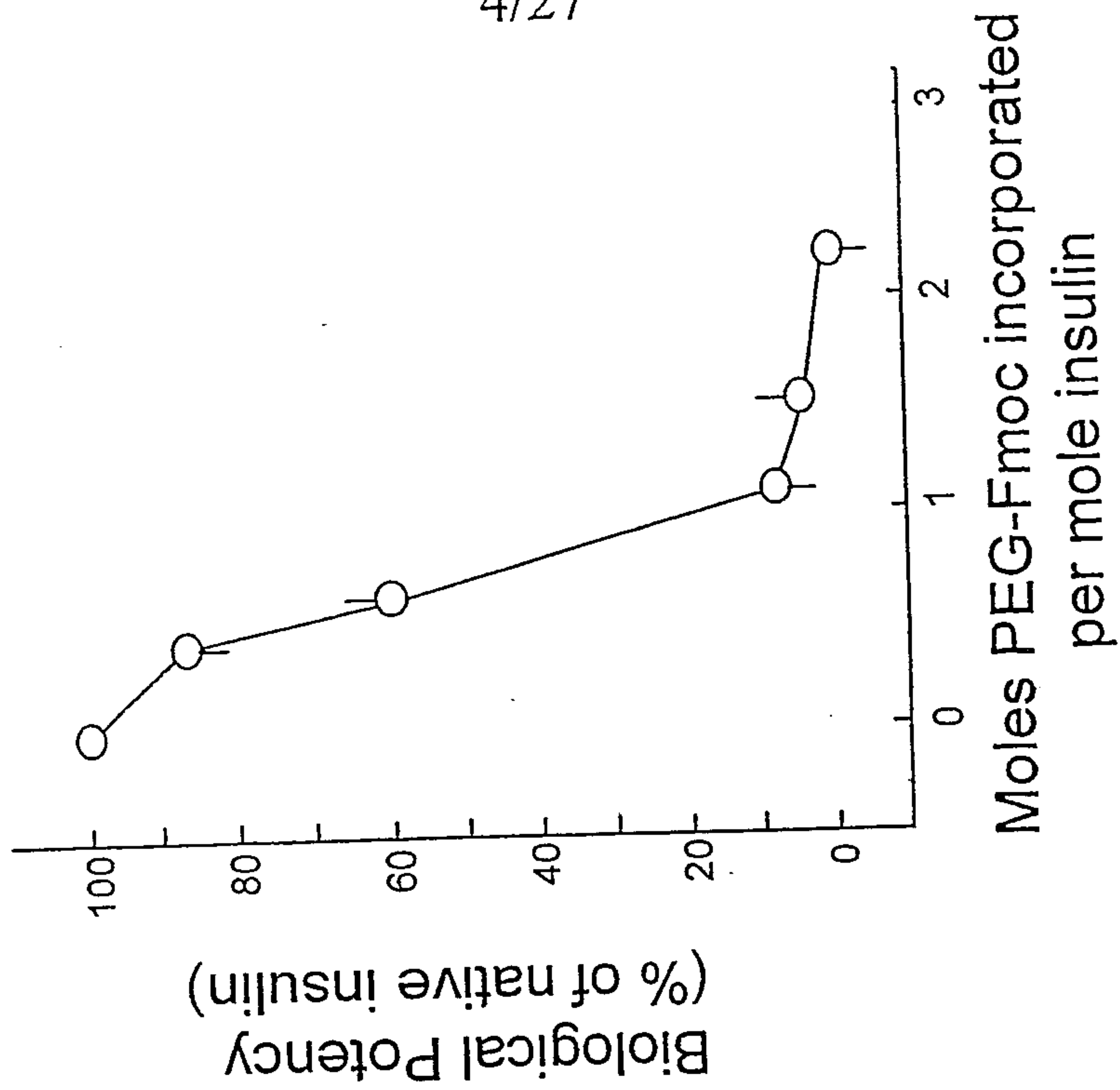


Fig. 4B

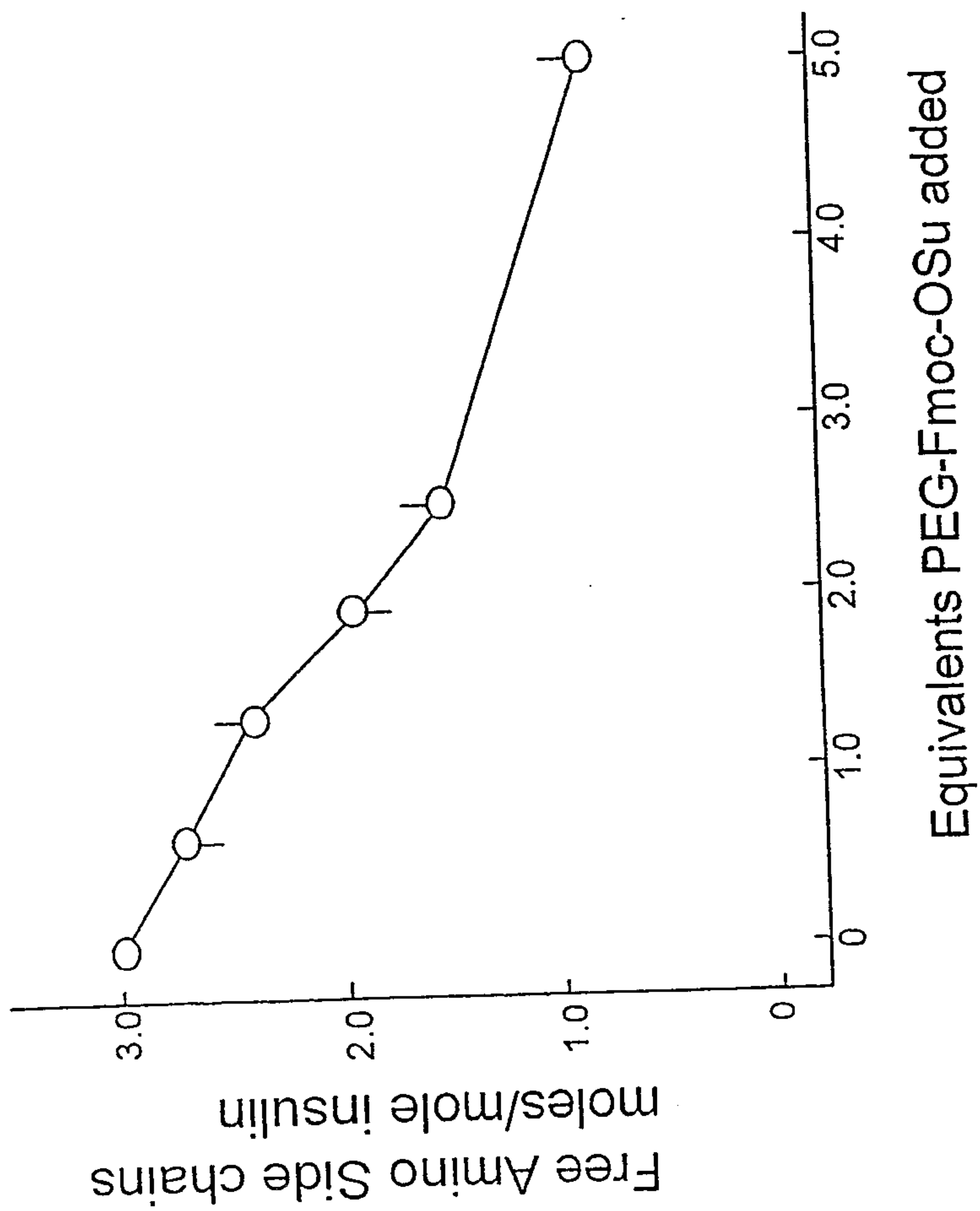


Fig. 4A

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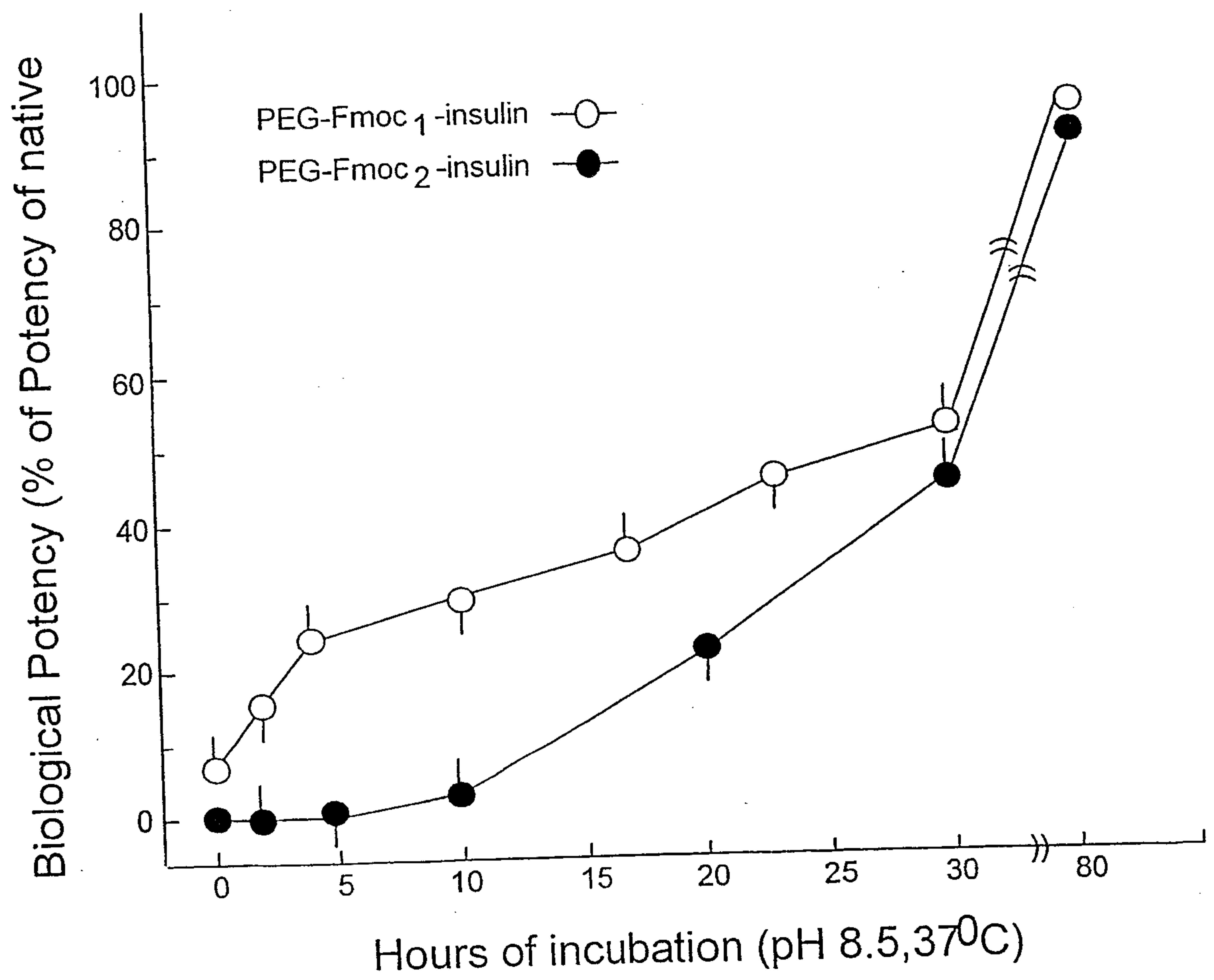


Fig. 5

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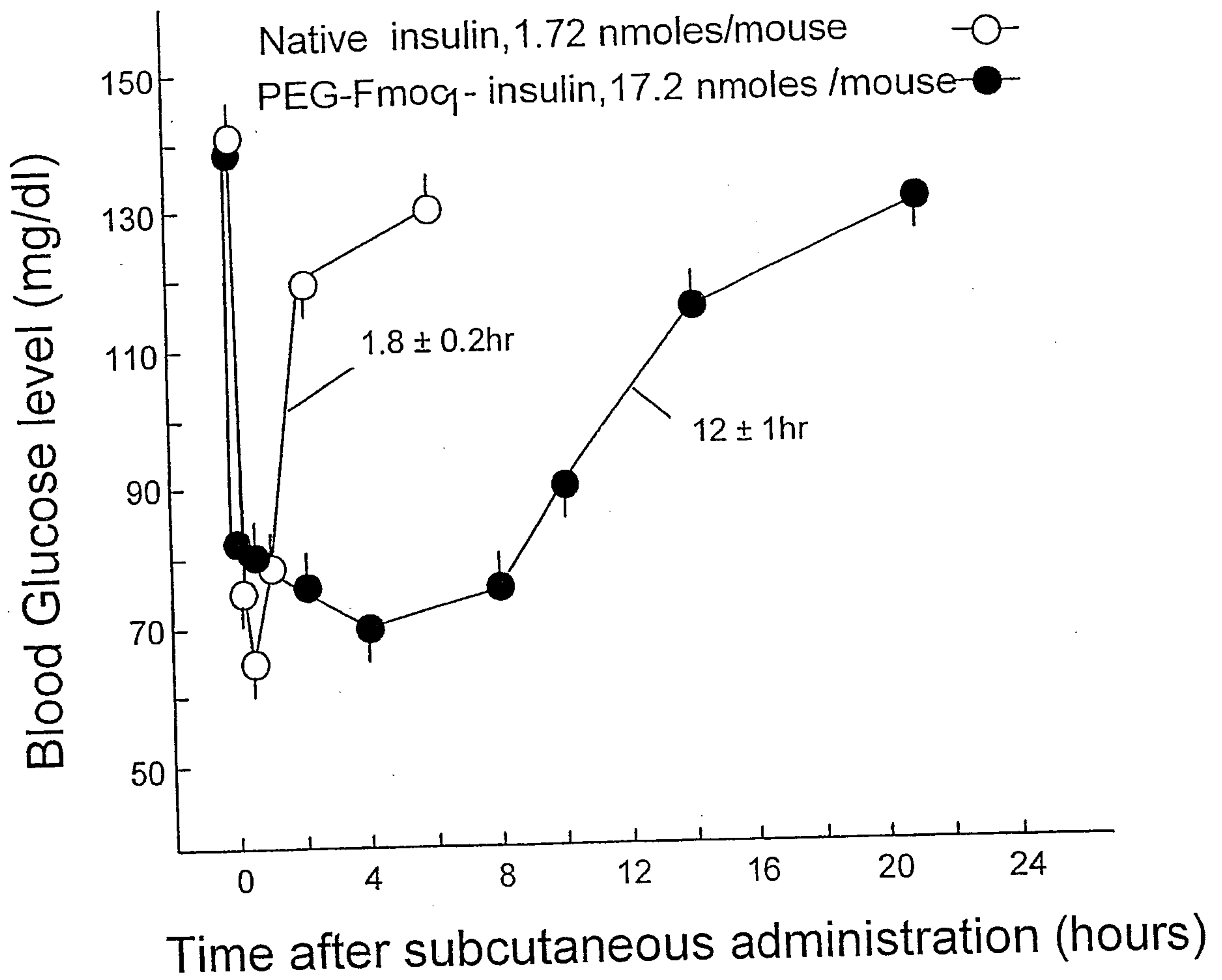
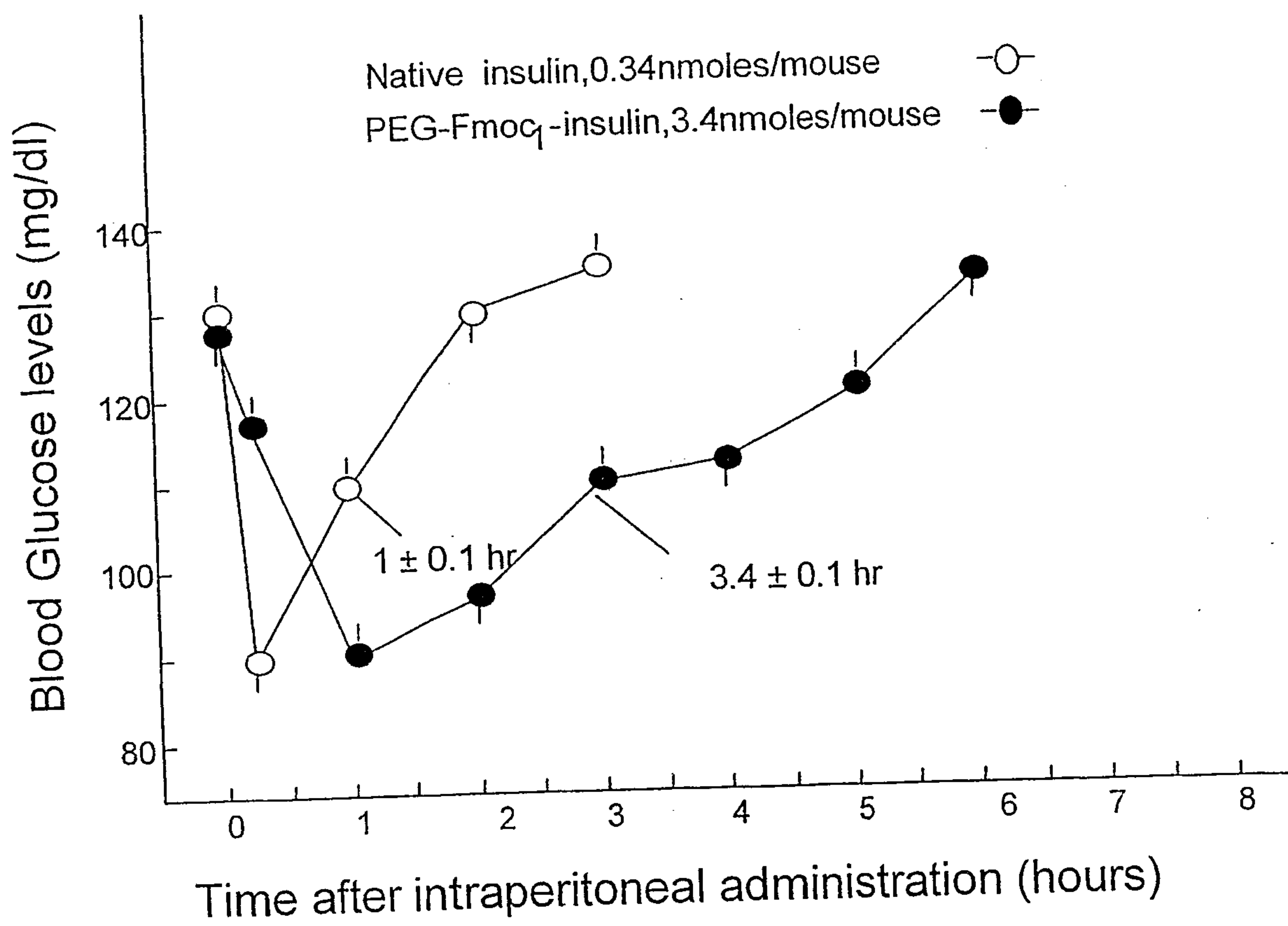


Fig. 6

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**Fig. 7**

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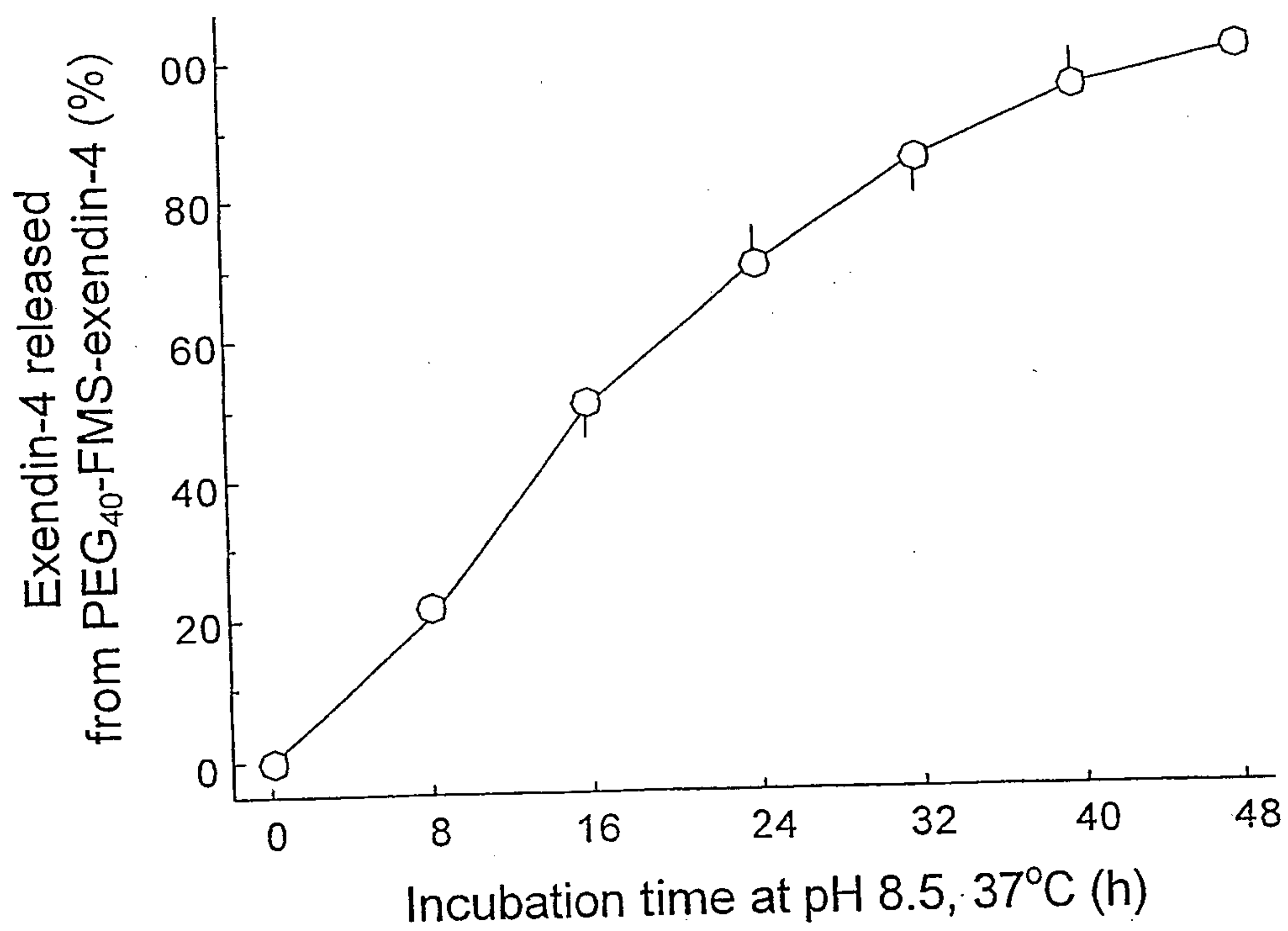


Fig. 8

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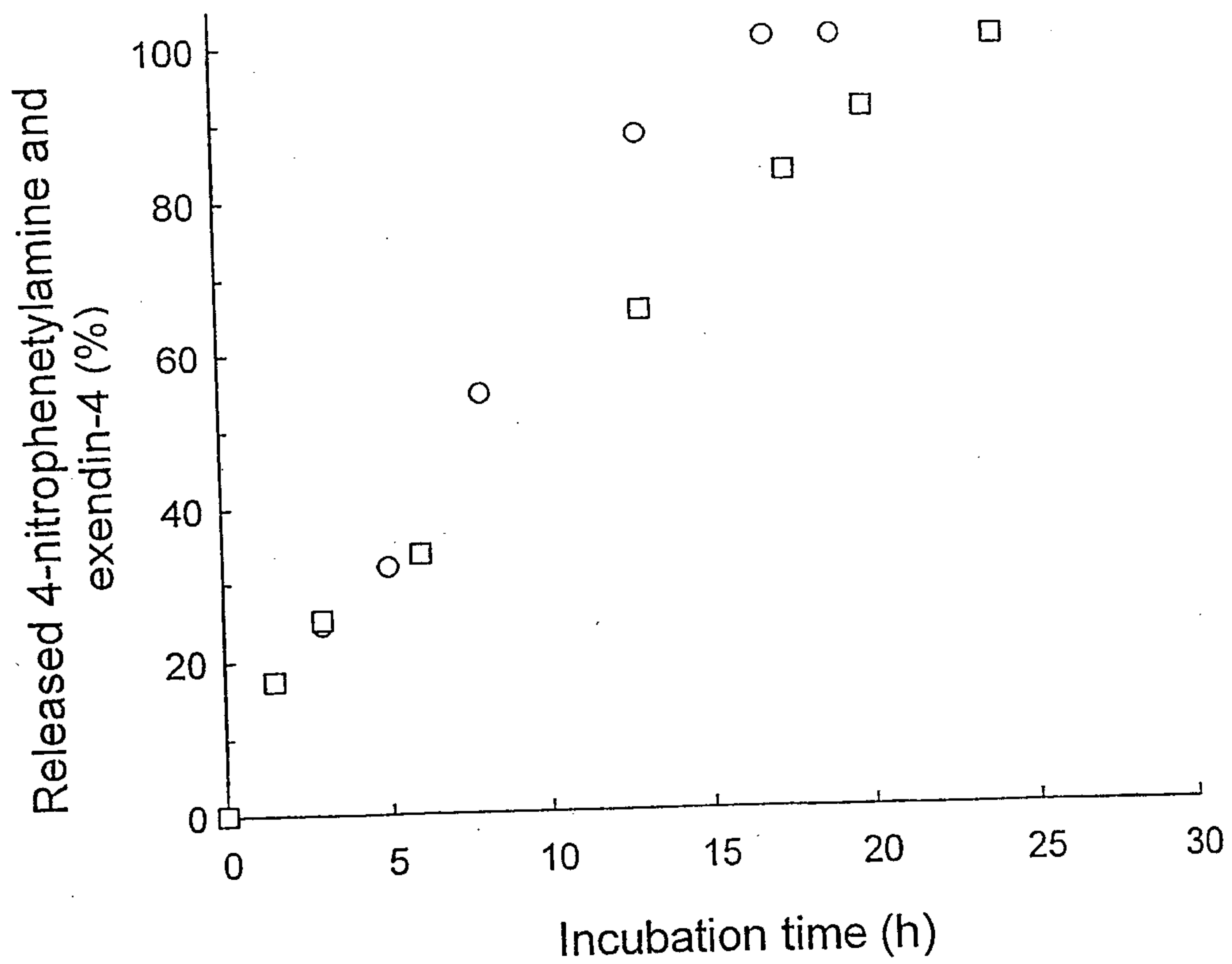


Fig. 9

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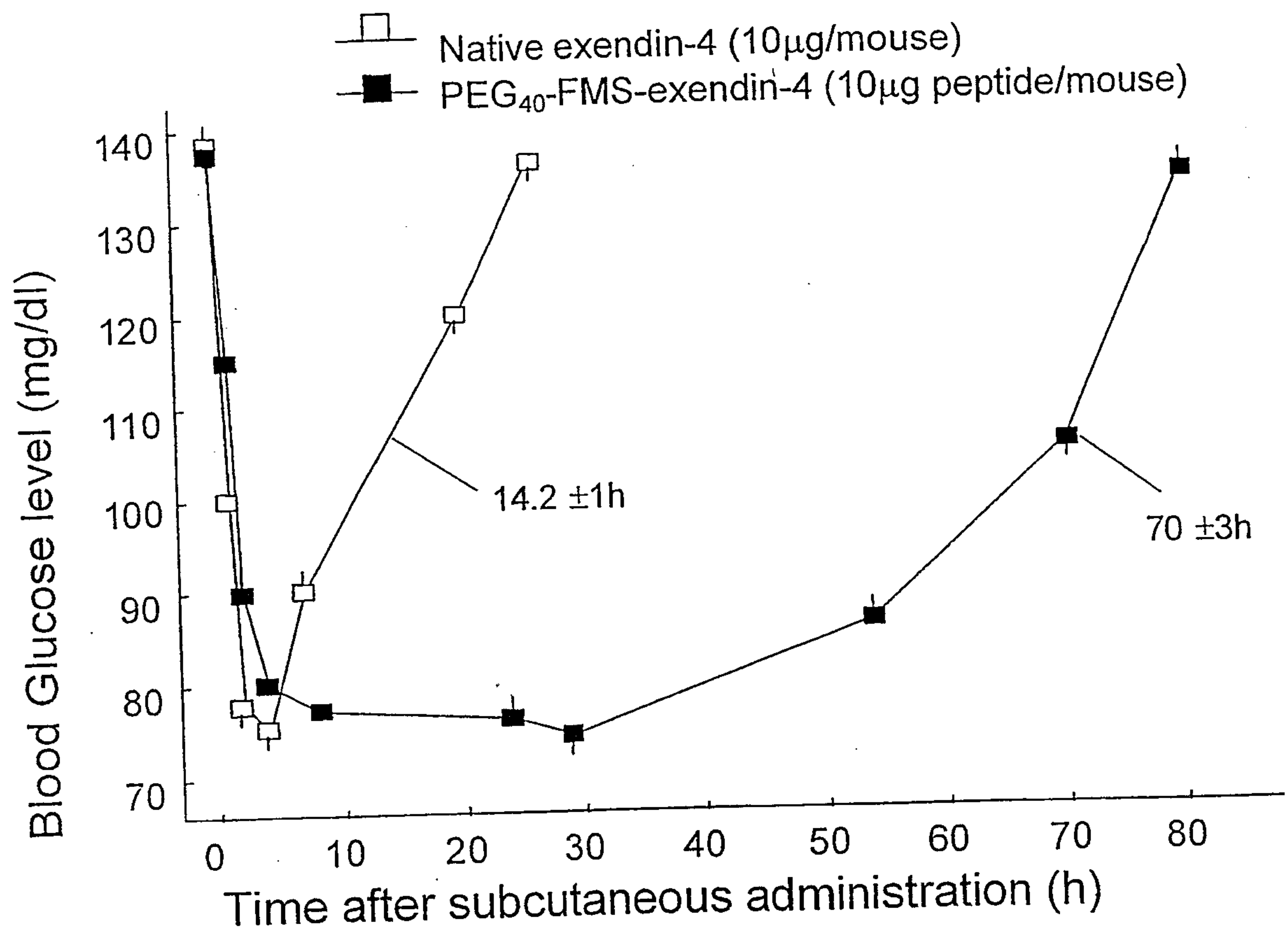


Fig. 10A

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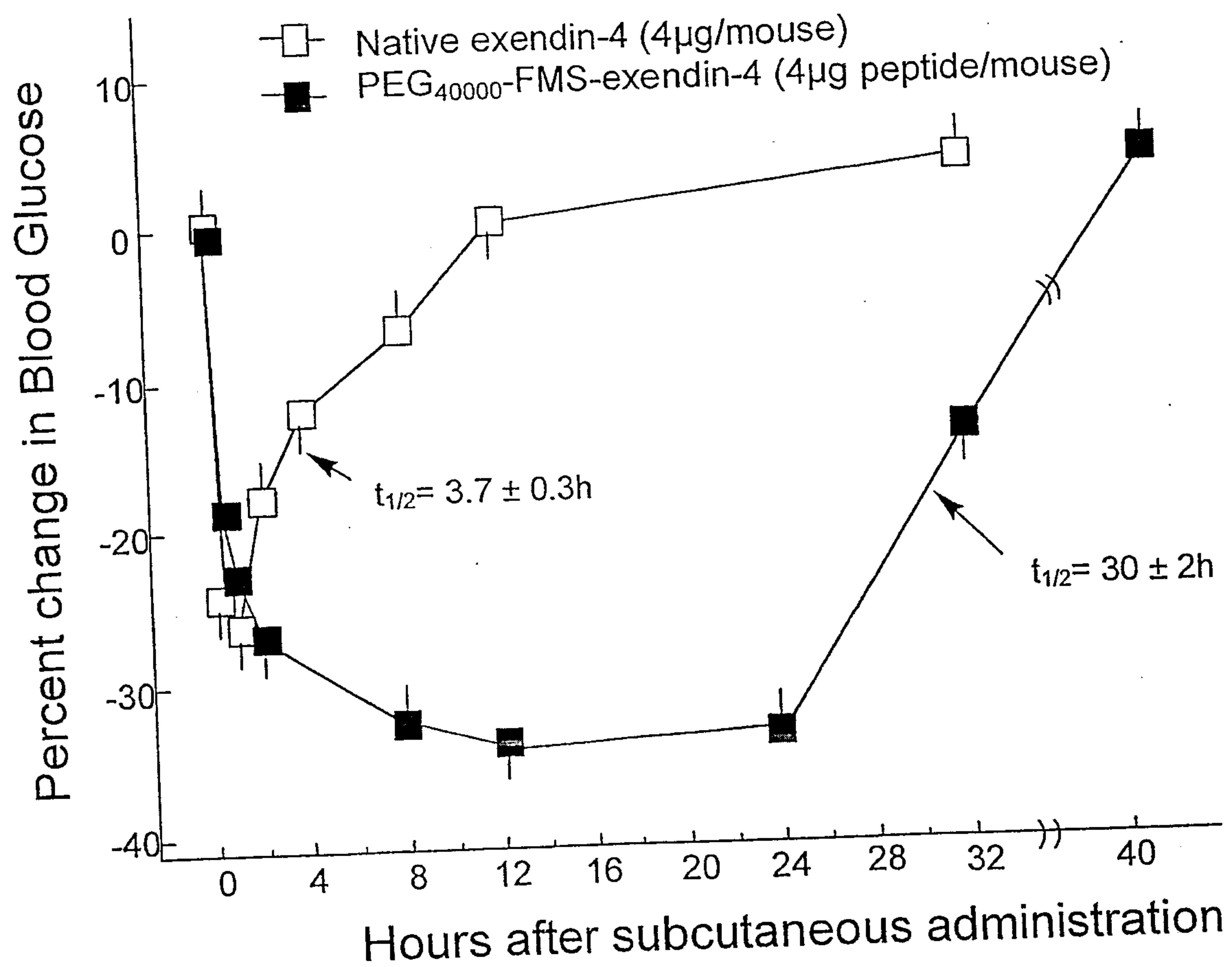


Fig. 10B

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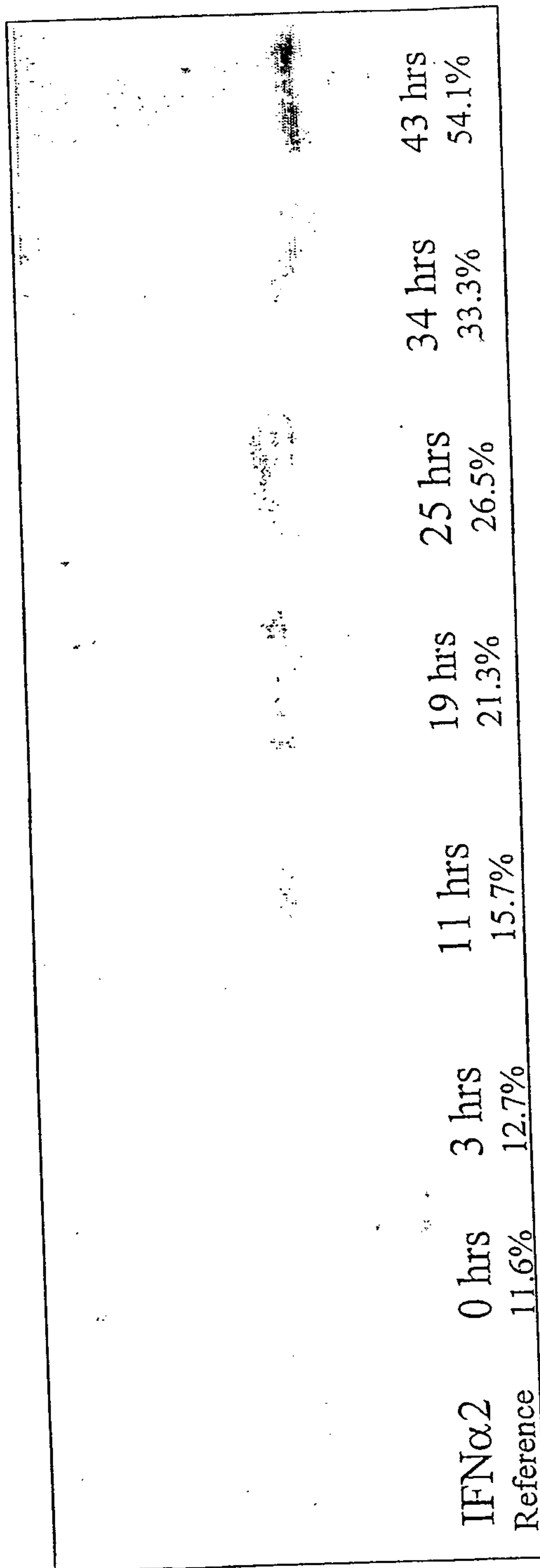
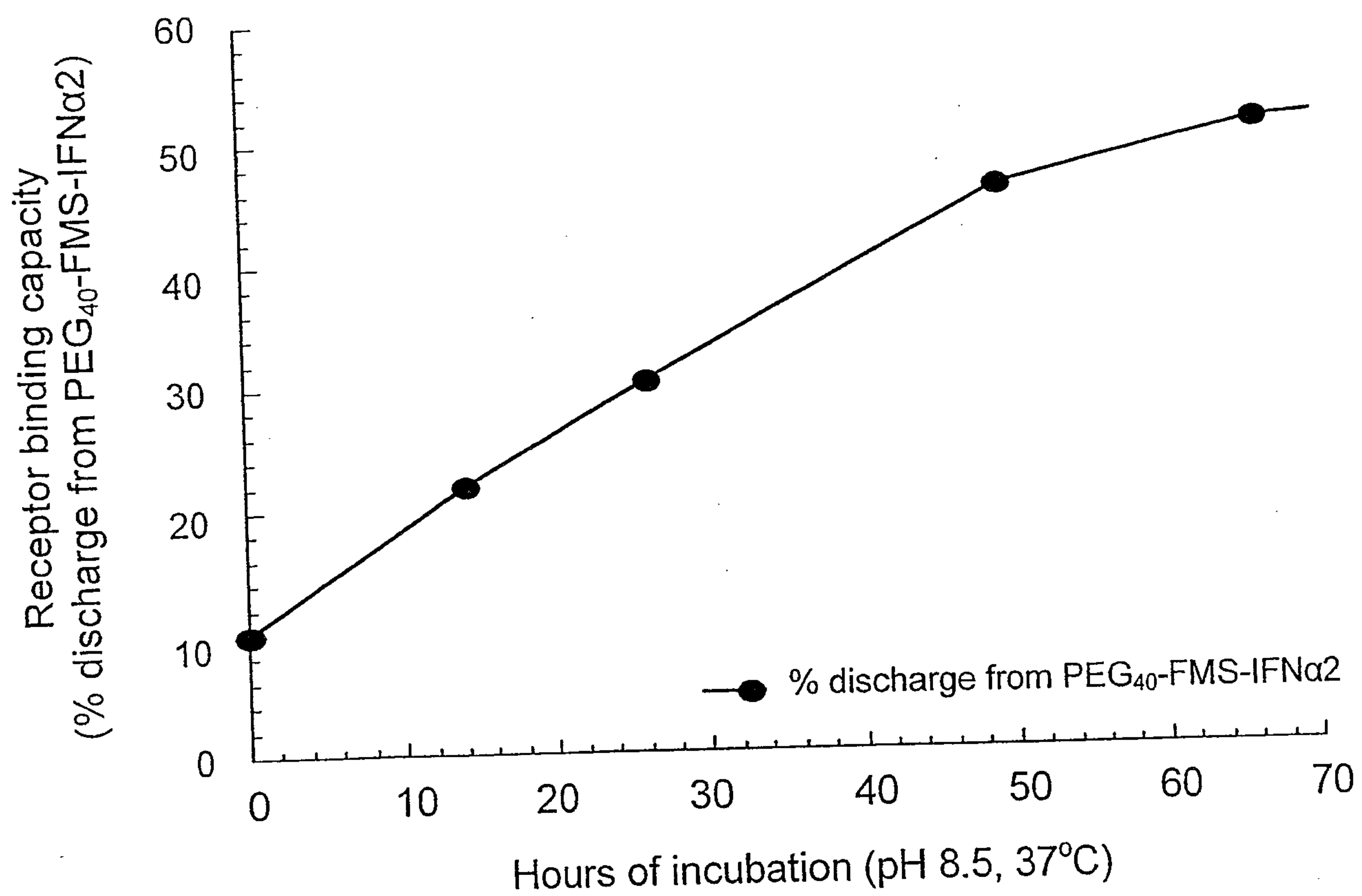


Fig. 11A

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**Fig. 11B**

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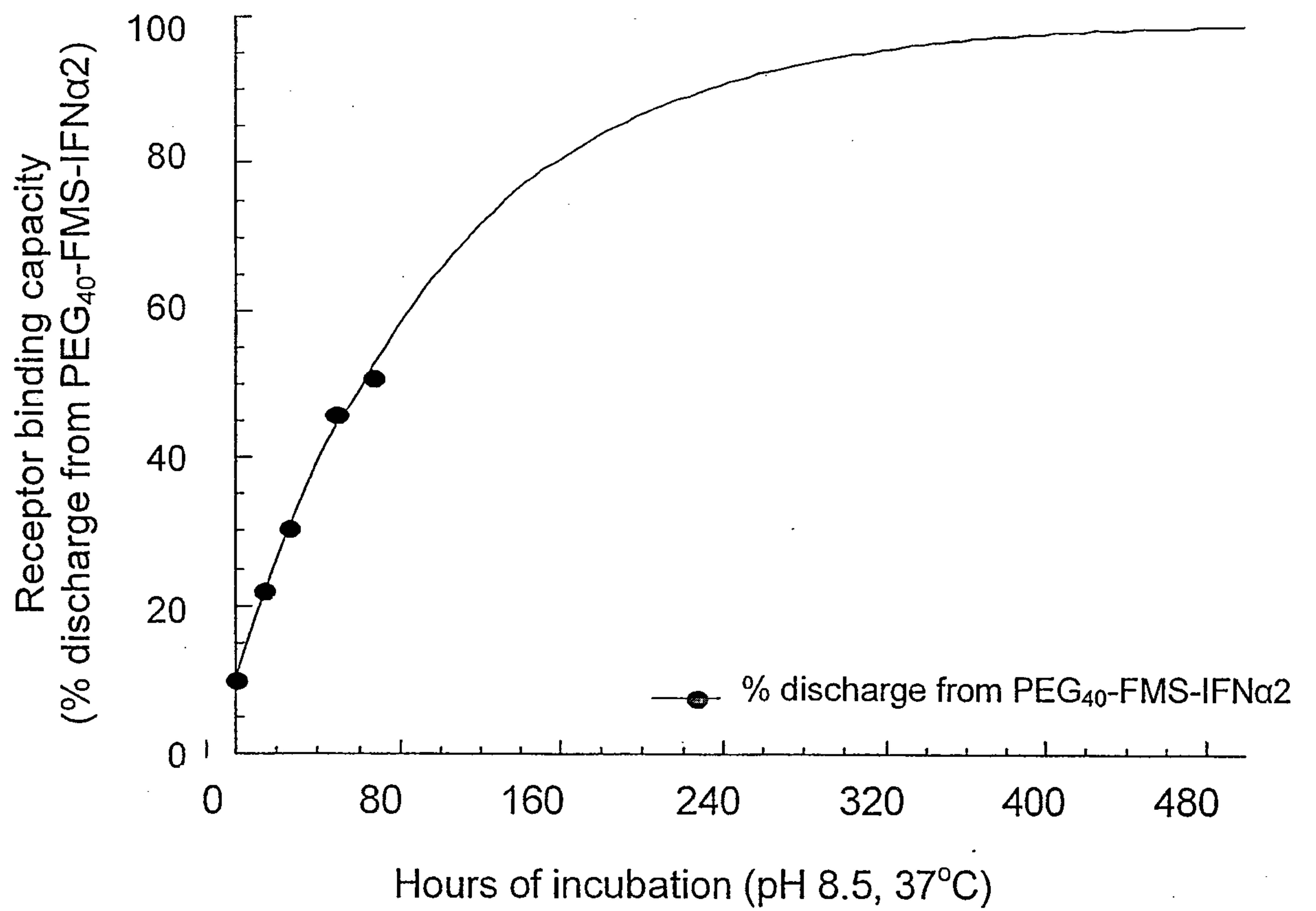


Fig. 11C

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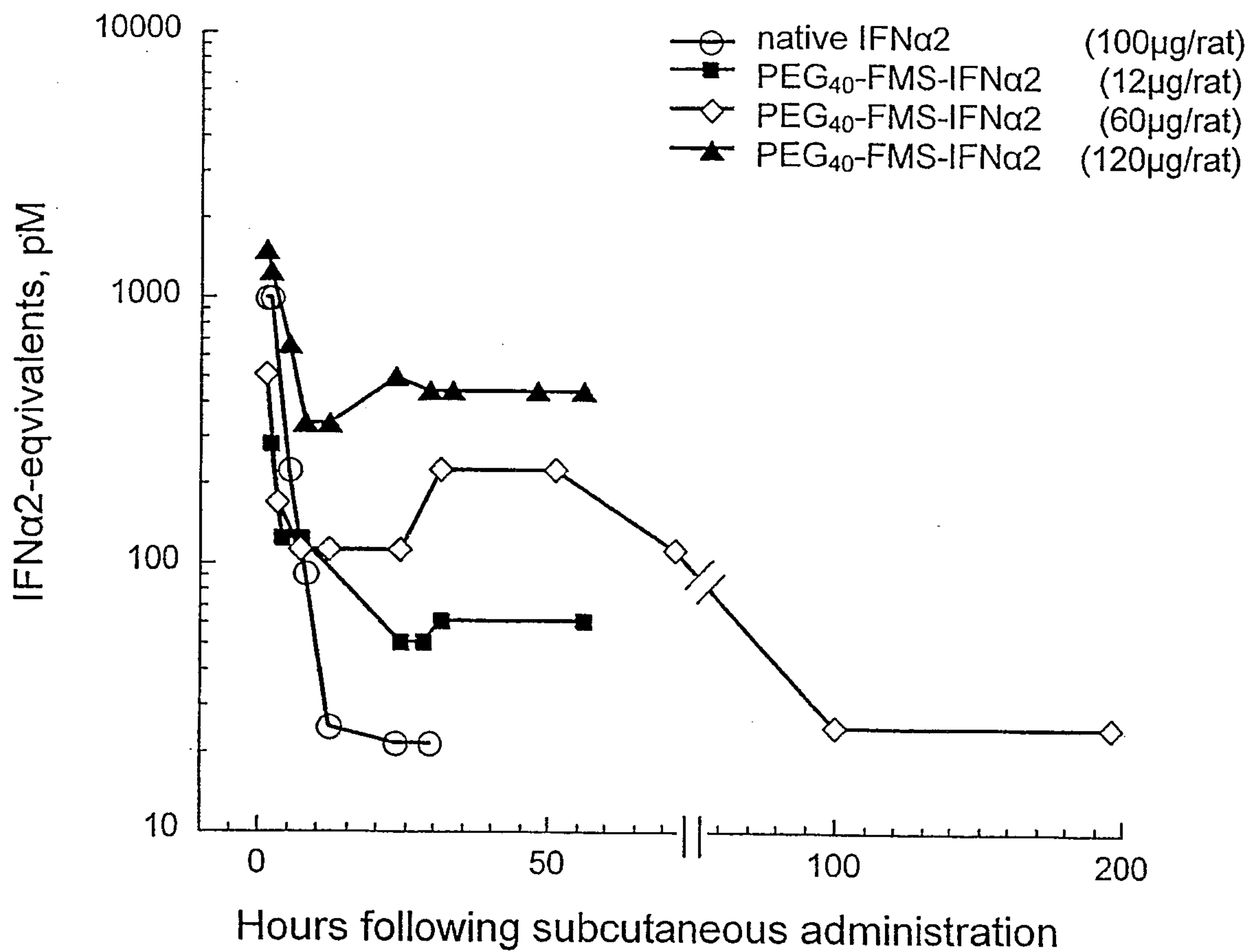


Fig. 12

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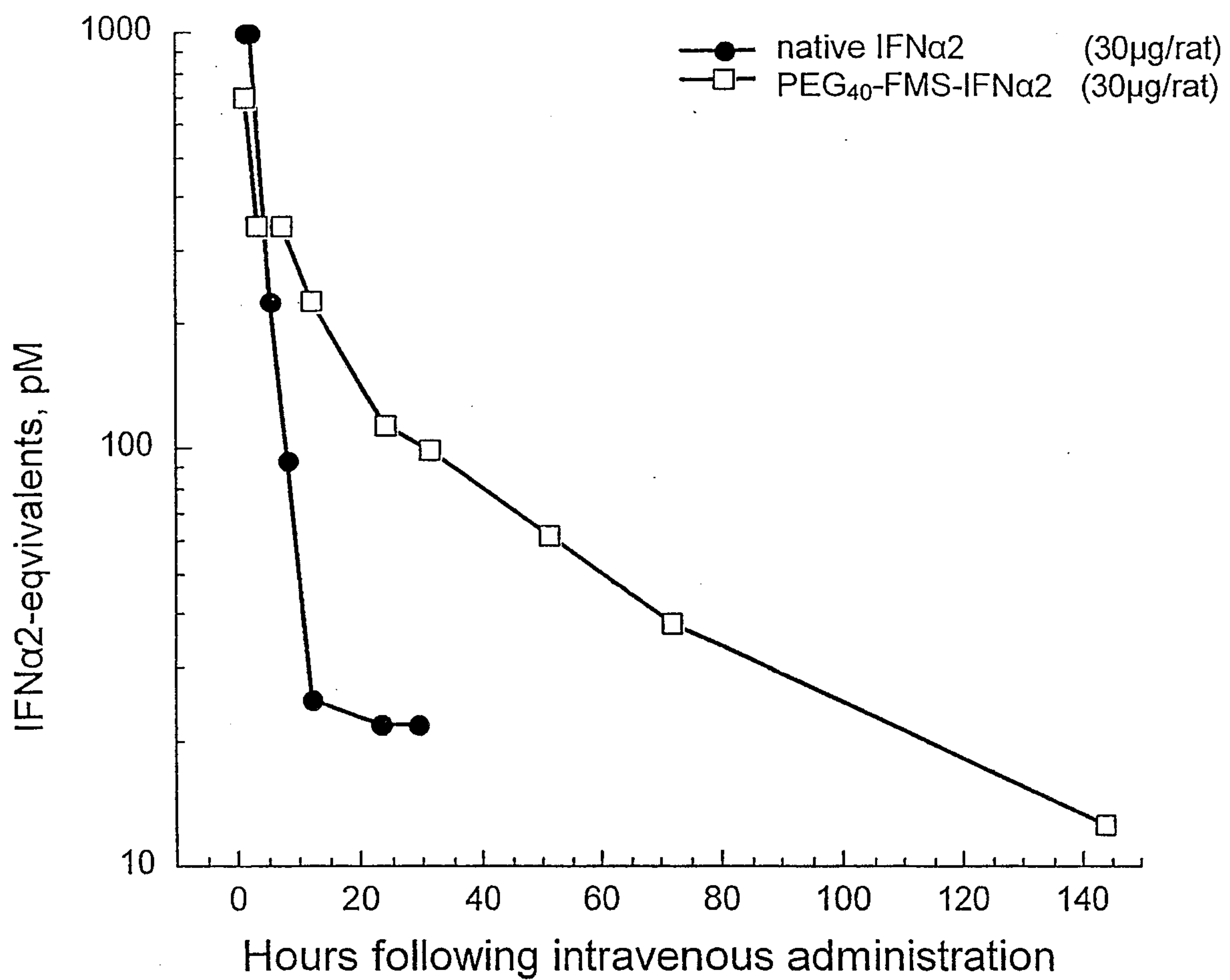


Fig. 13

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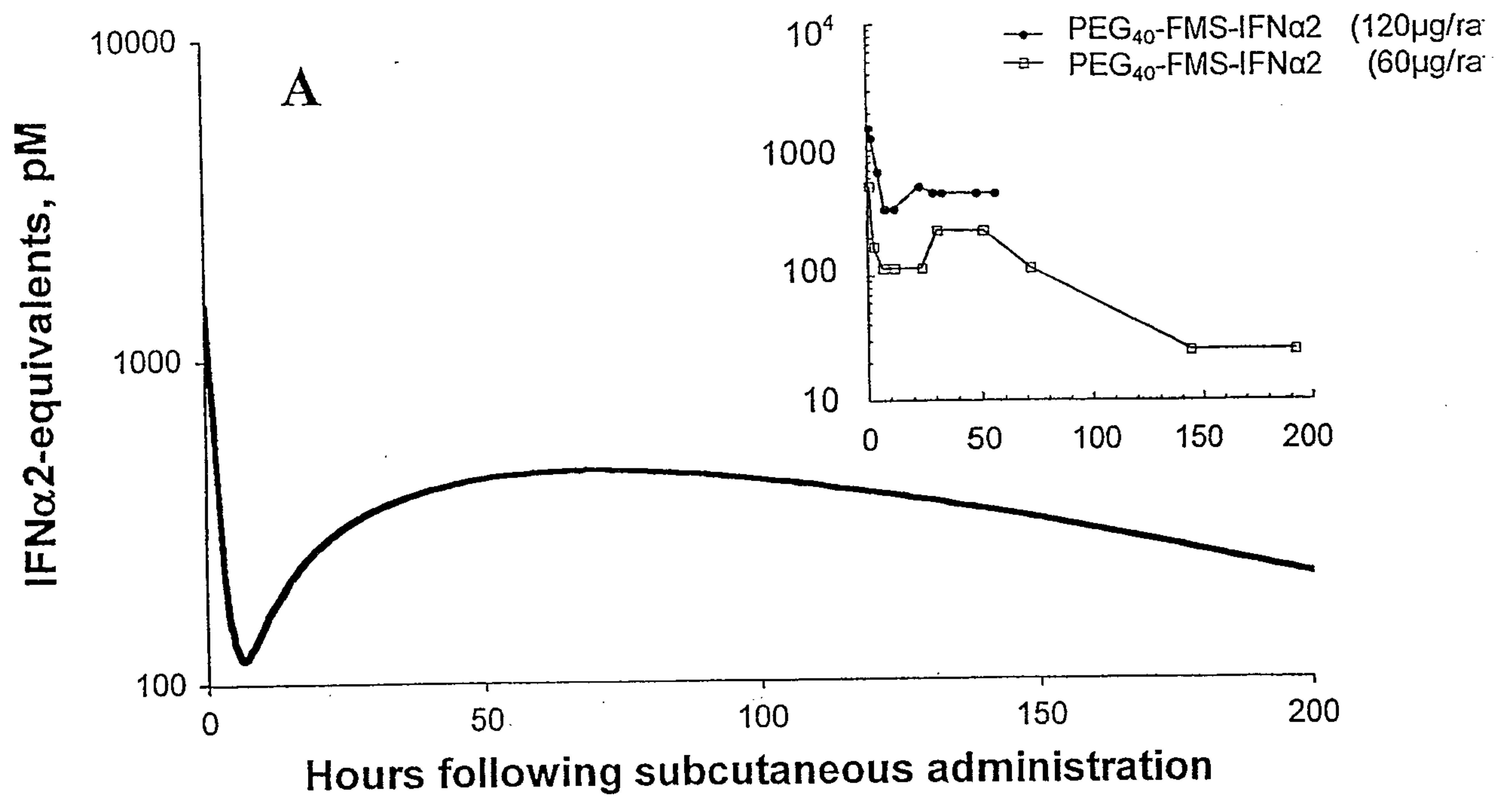


Fig. 14A

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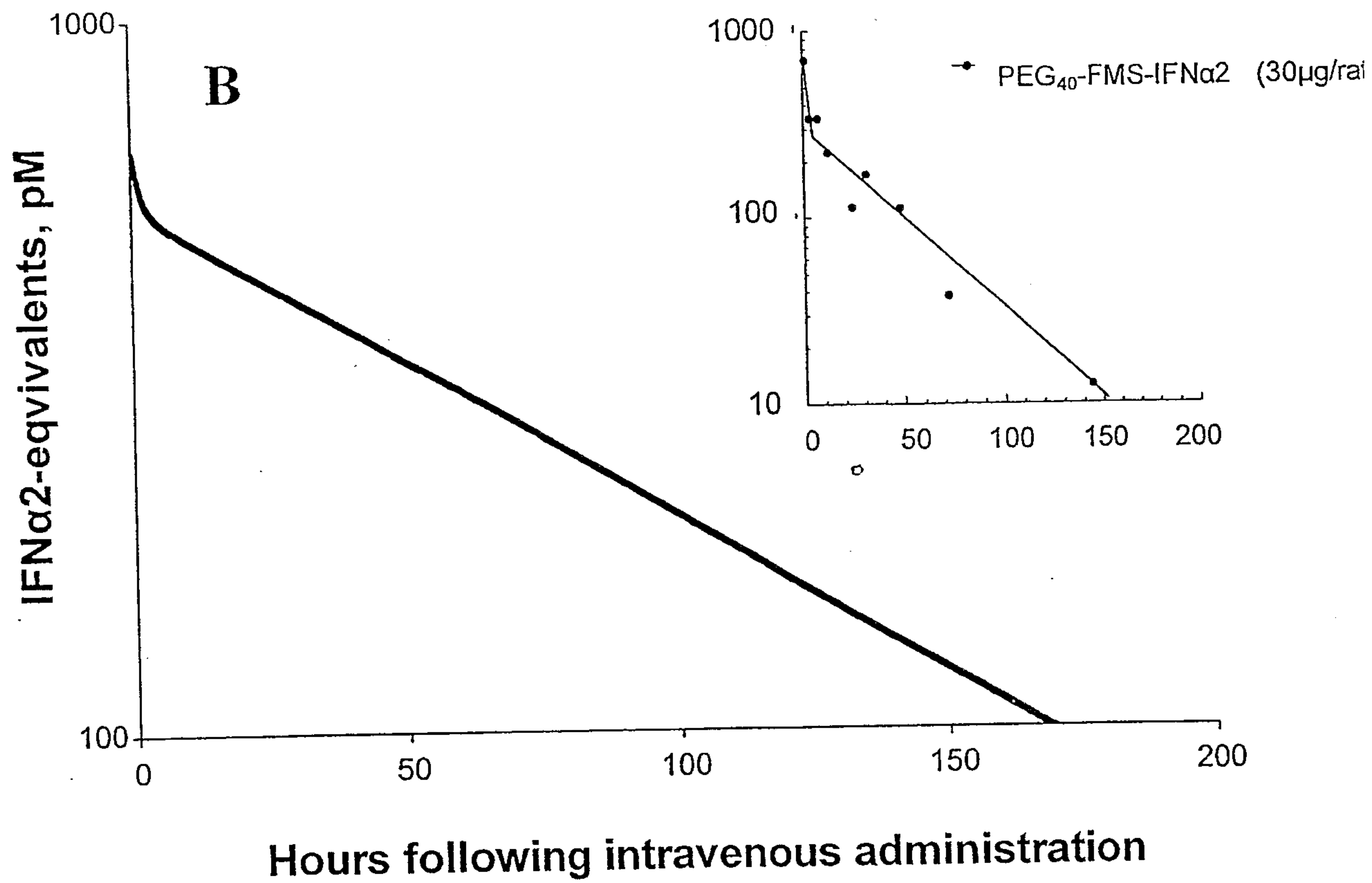


Fig. 14B

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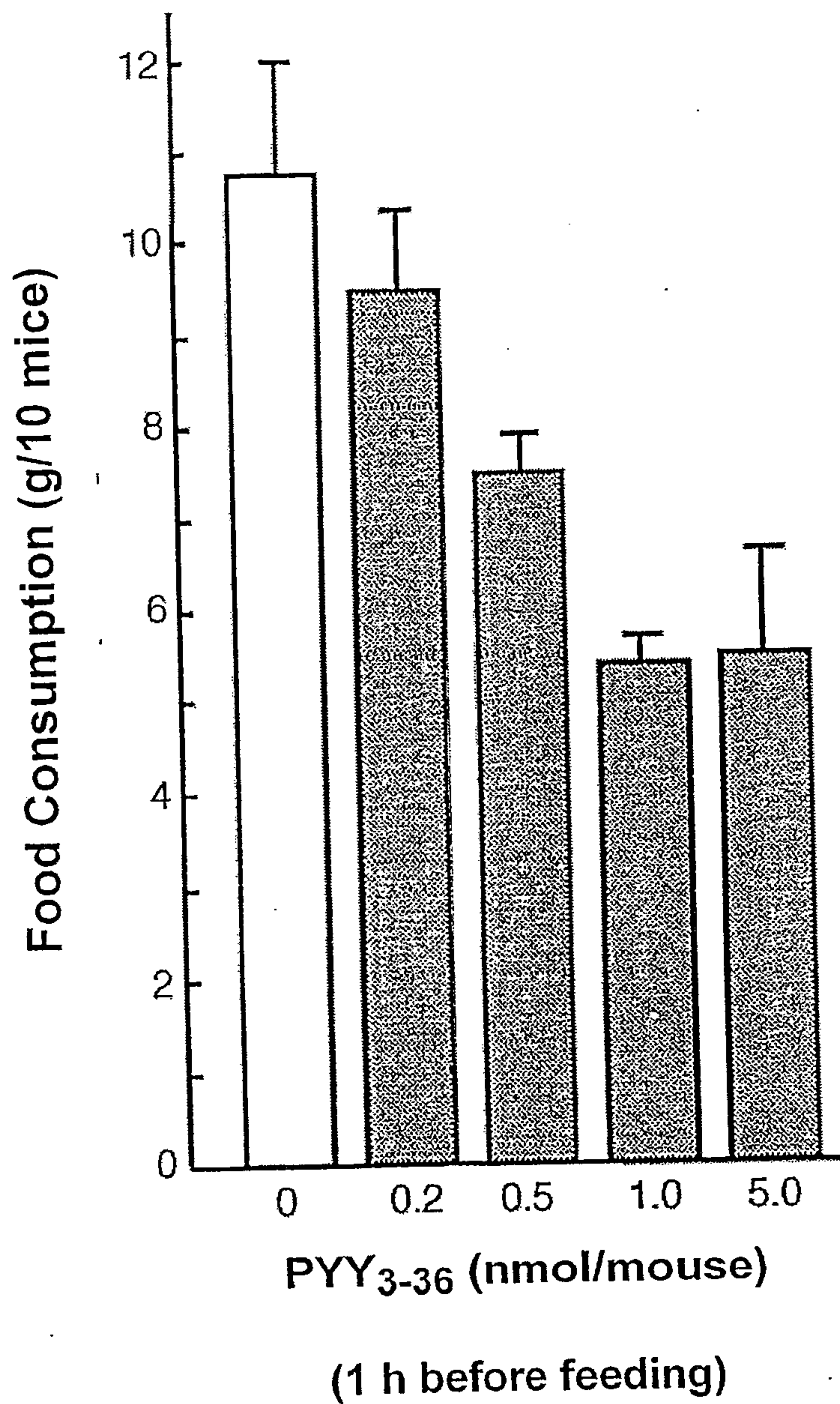


Fig. 15

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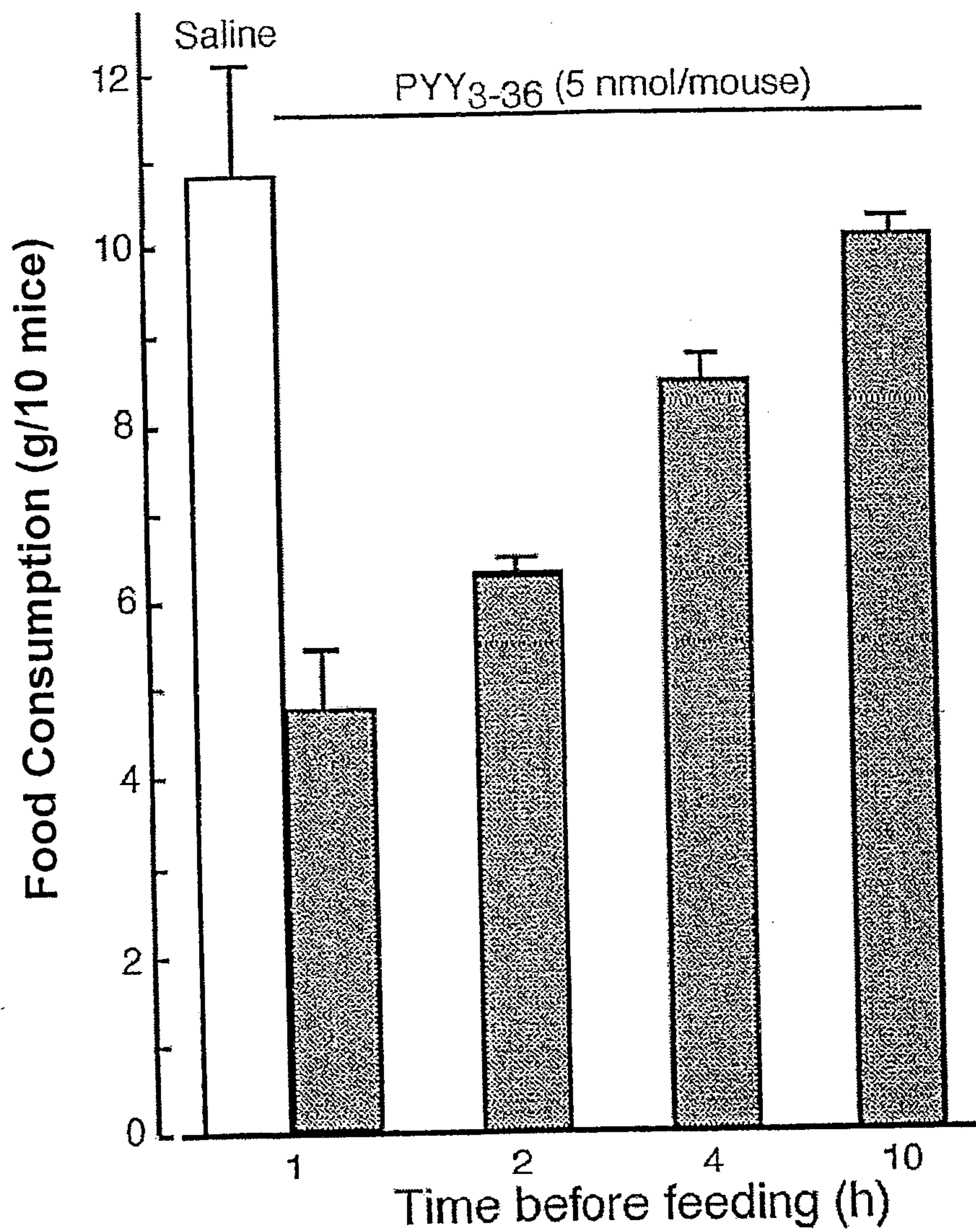


Fig. 16

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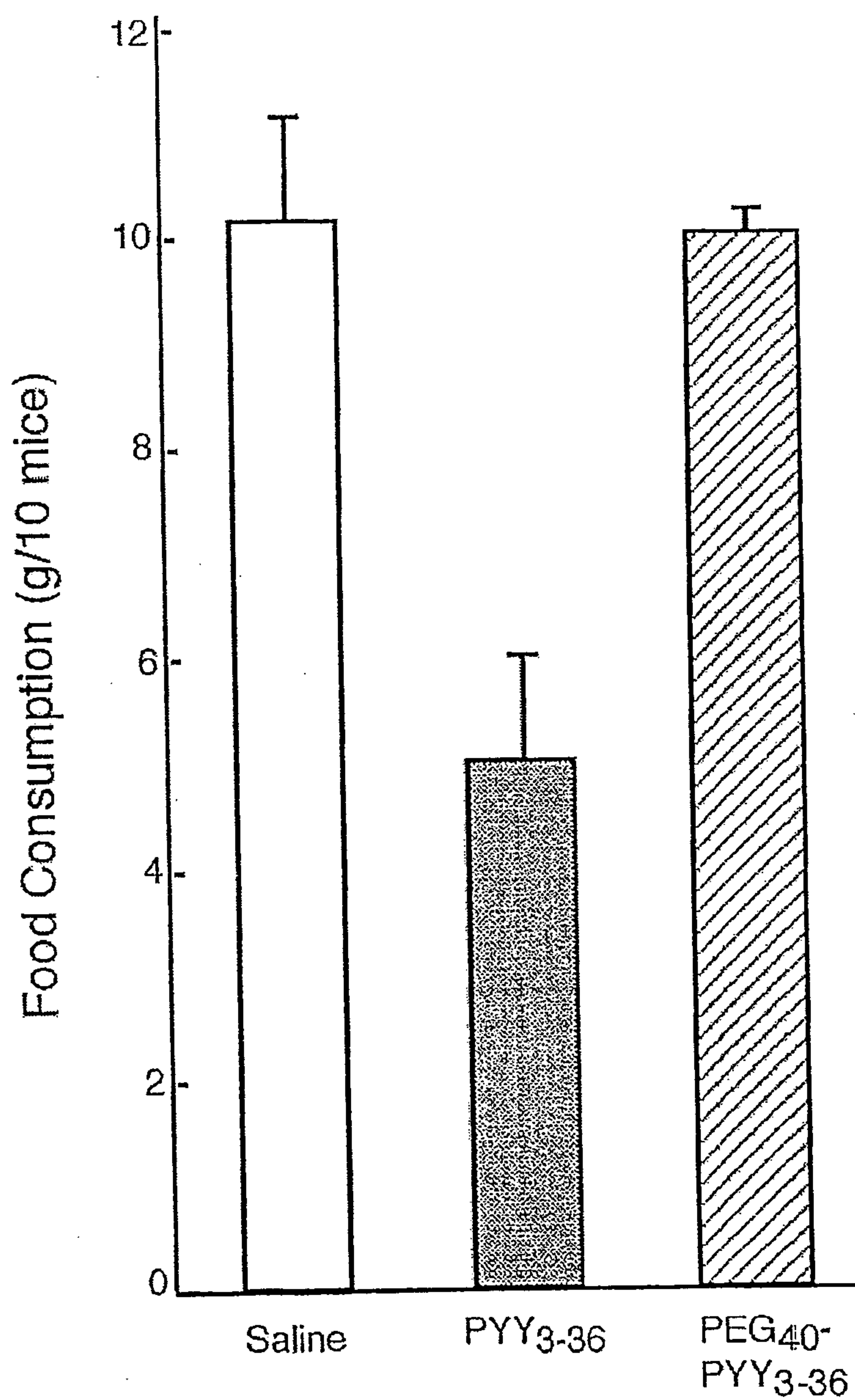


Fig. 17

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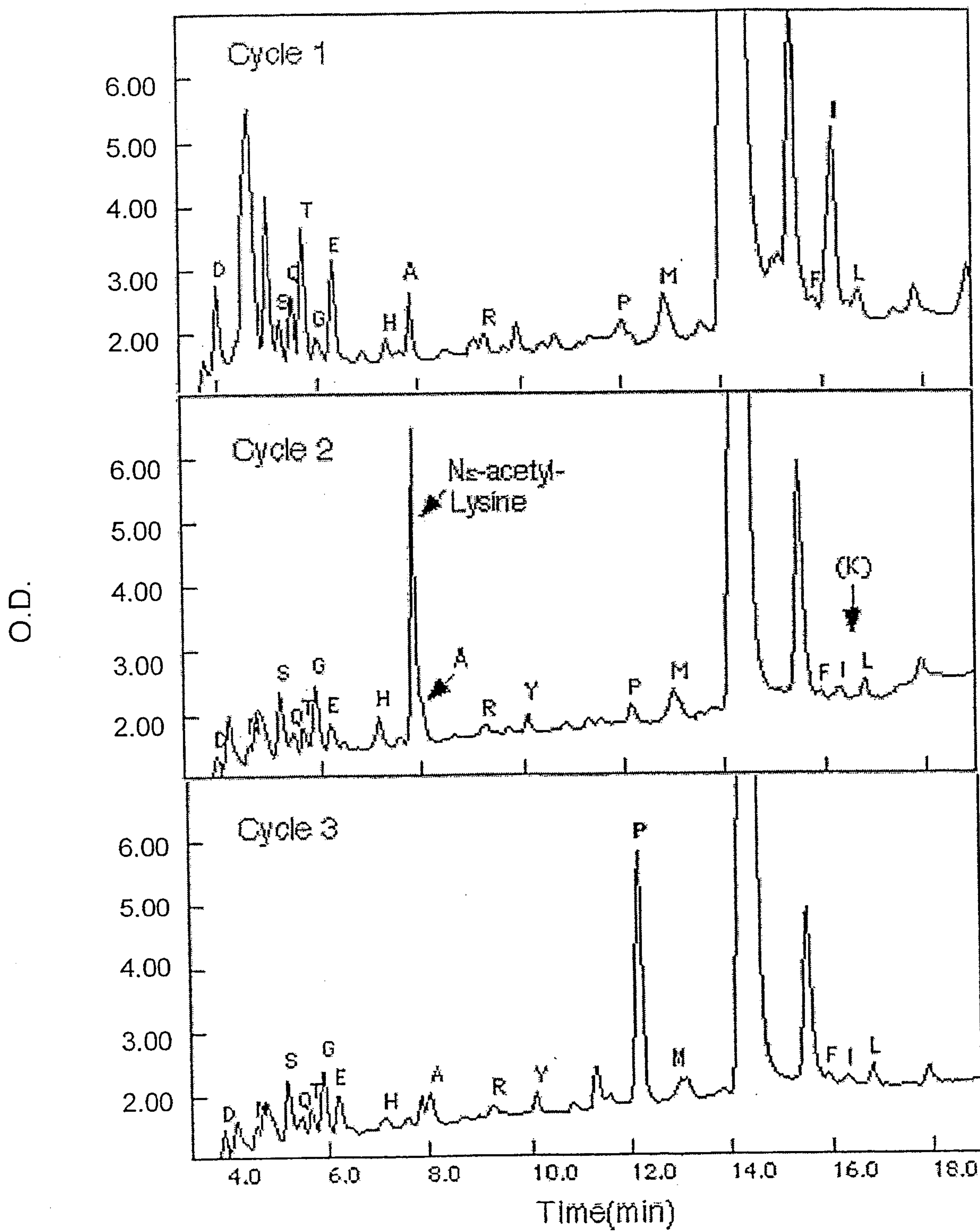


Fig. 18

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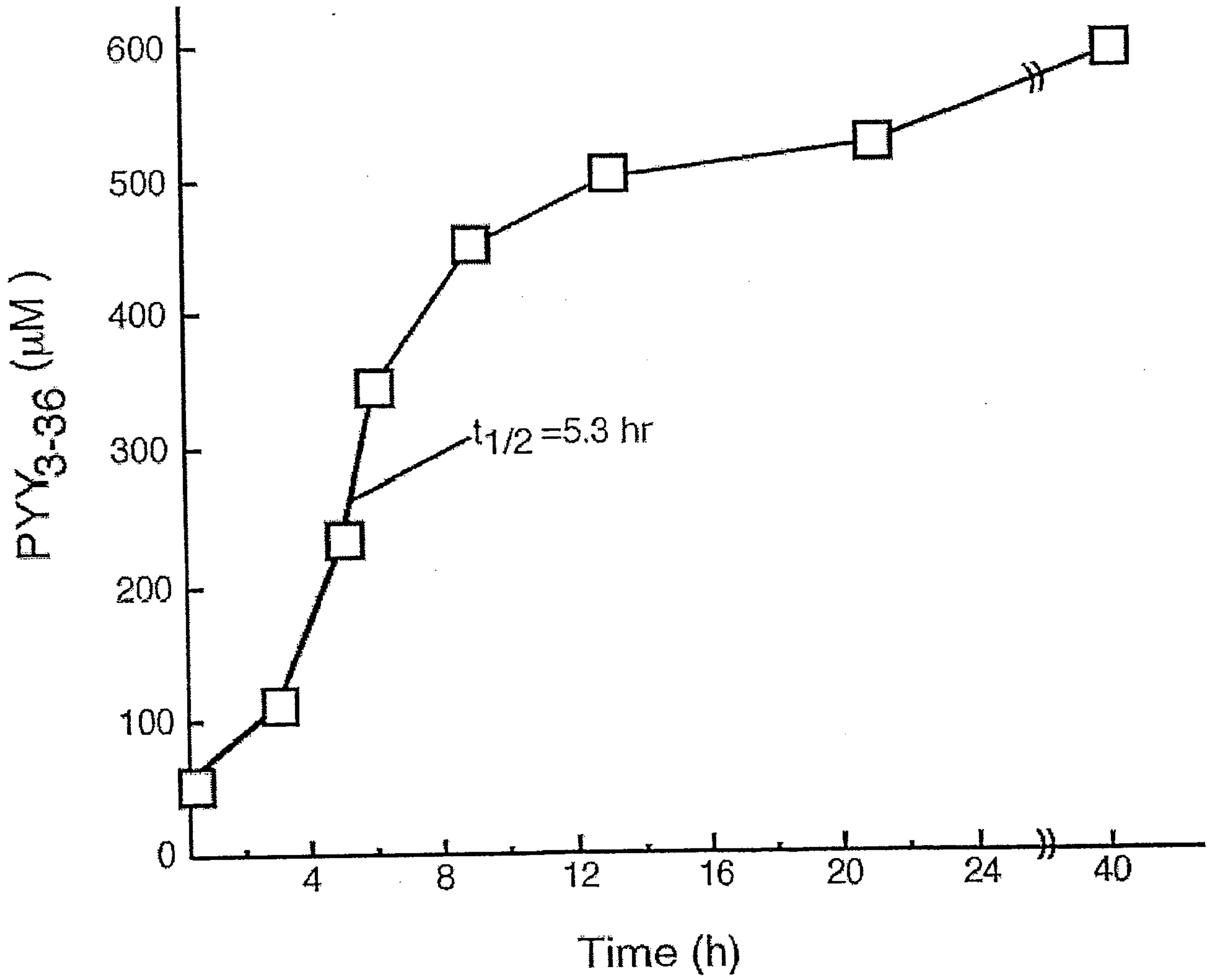


Fig. 19

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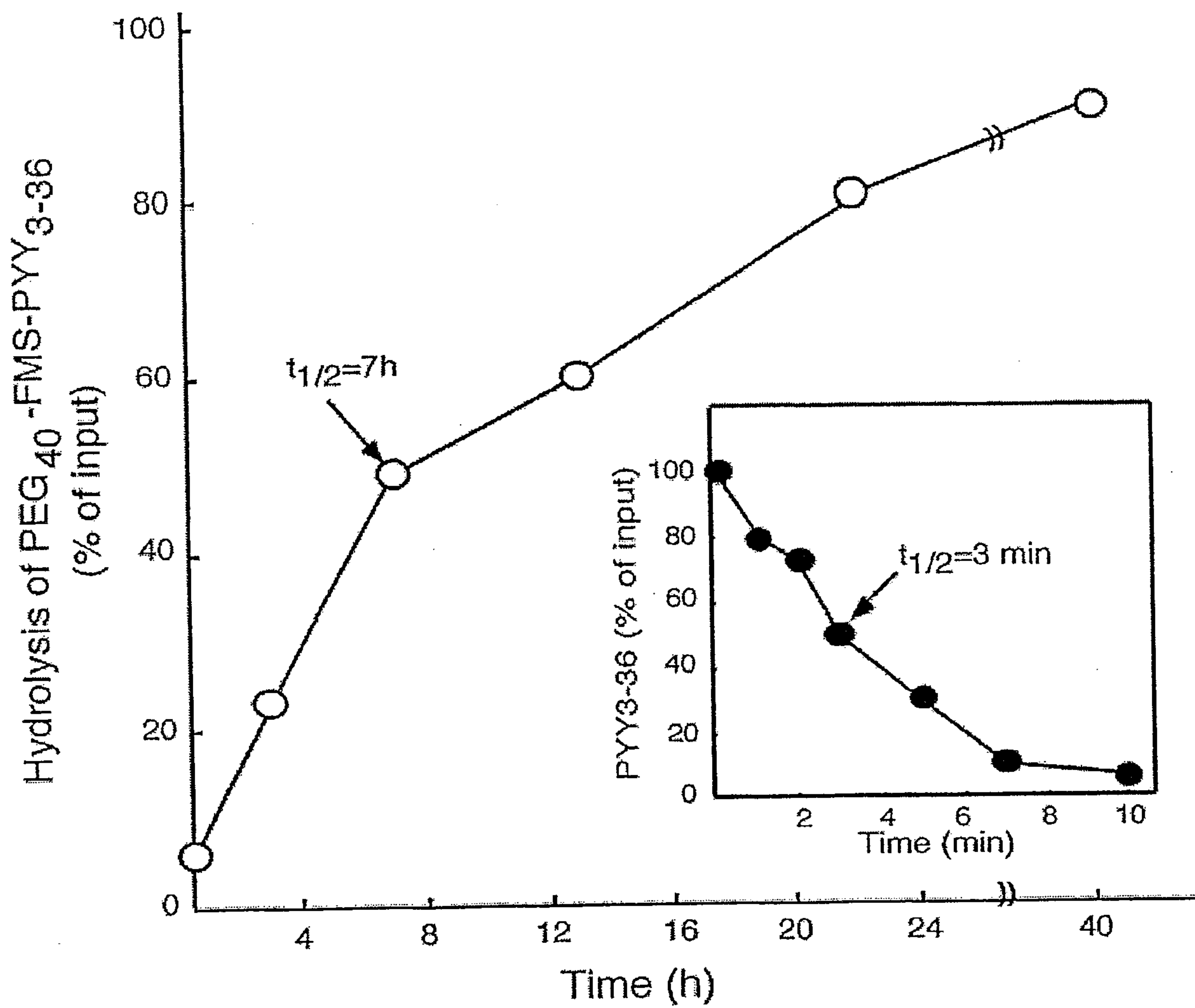


Fig. 20

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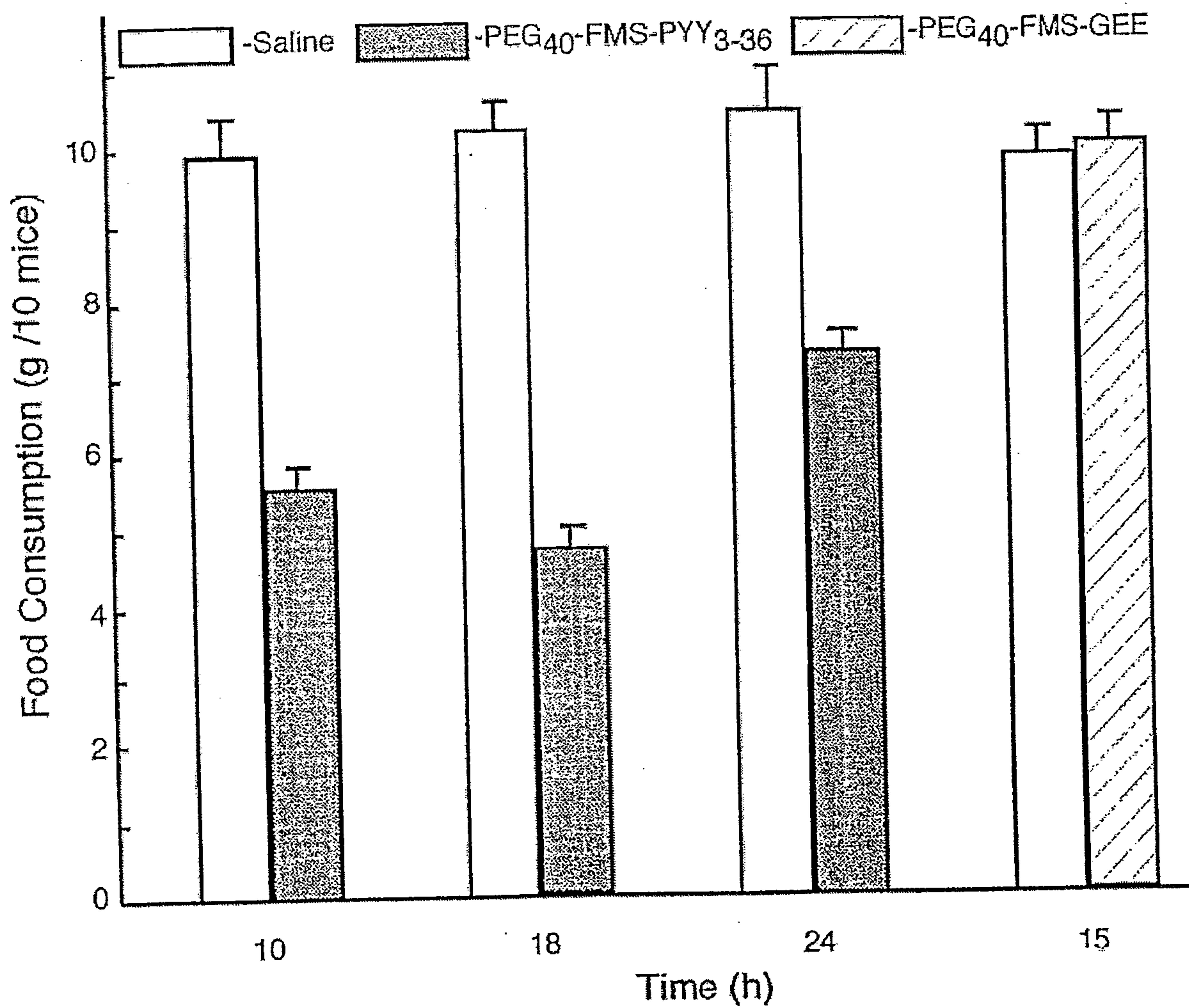


Fig. 21

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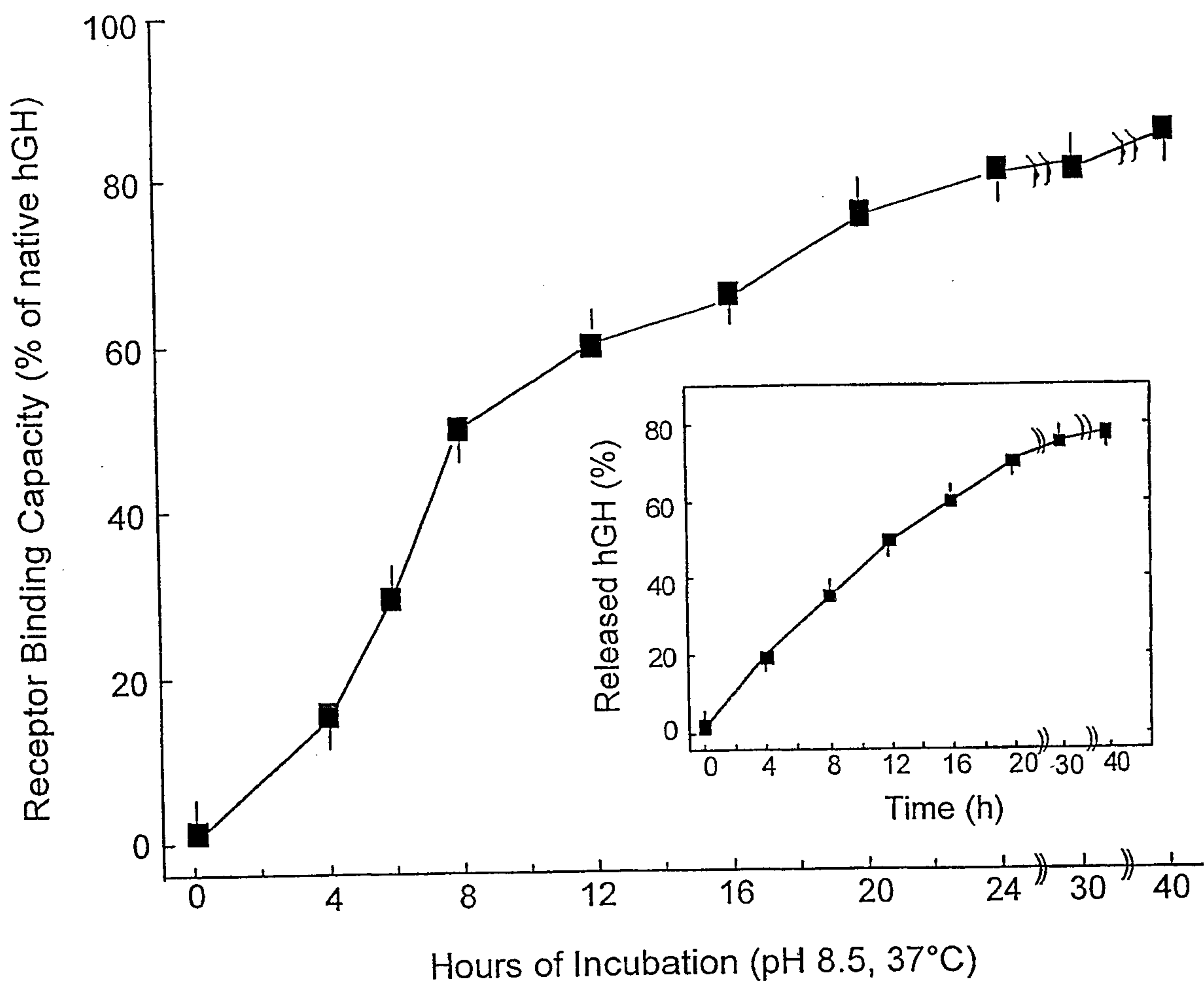


Fig. 22

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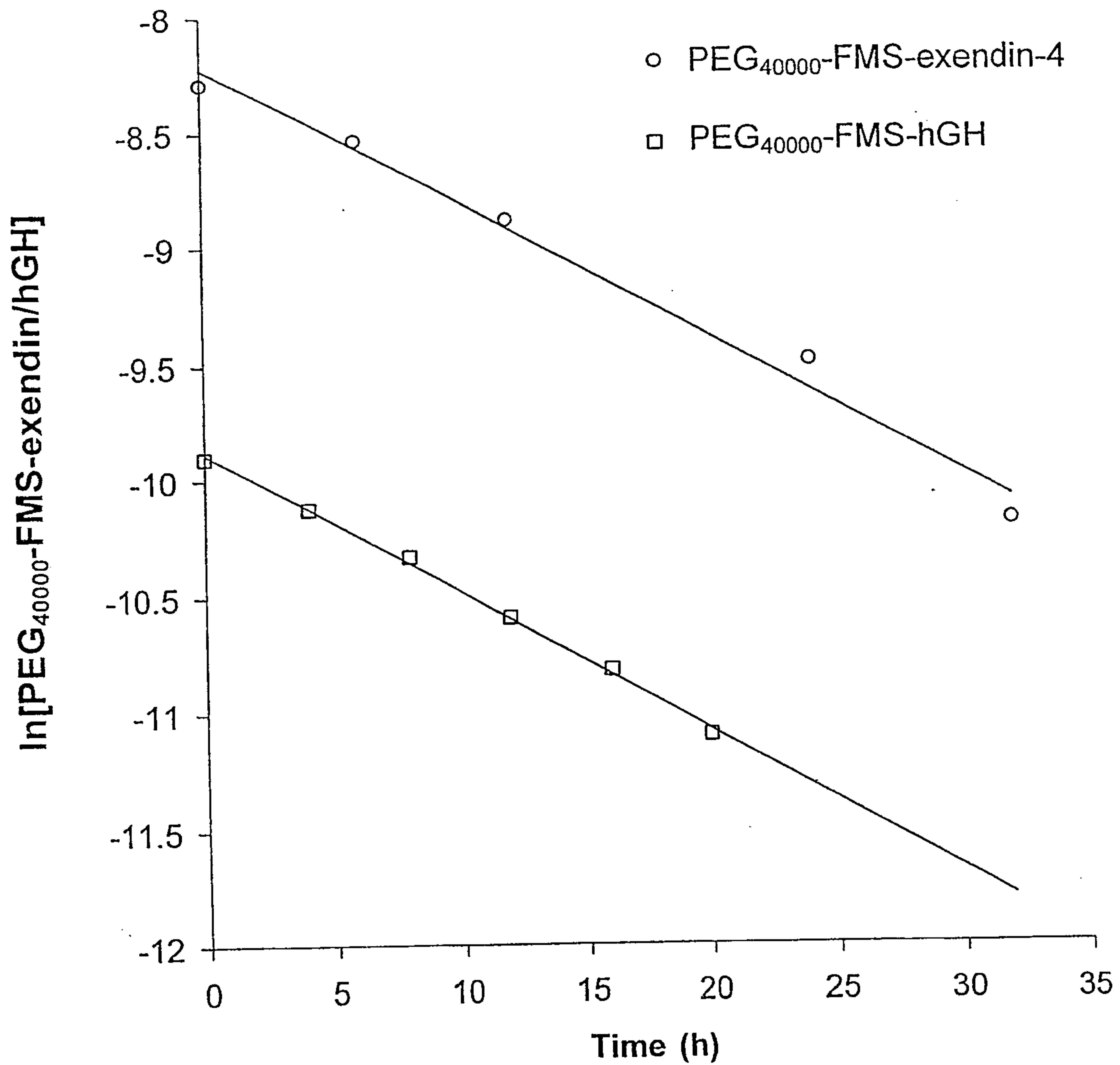


Fig. 23