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(54) Title: ACTIVATABLE PHOTODYNAMIC THERAPY AGENTS

(57) **Abstract:** The present invention relates to the field of conjugates comprising a first substrate which is attached to a least one photosensitizer and at least ont first quencher, and methods for their use. More particularly, the present invention relates to photodynamic therapy agents. The invention further relates to methods for decontaminating blood and methods for treating cancer or viral infection in a subject using the conjugates of the present invention.

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ACTIVATABLE PHOTODYNAMIC THERAPY AGENTS

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to the field of photodynamic therapy agents. The invention further relates to compositions and methods for decontaminating blood and treating disease such as cancer or viral infection in a subject using the photodynamic therapy agents of the present invention.

Background Art

Photodynamic Therapy

Photodynamic therapy (PDT) is a minimally invasive treatment modality for many diseases, such as cancers and age-related macular degeneration. It has also been one of the very few sterilization methods that may be applied to red blood cells (RBCs). PDT involves the combination of light and a photosensitizer, typically a porphyrin derivative. Each factor is harmless by itself, but when combined with oxygen (O_2), lethal reactive oxygen species are produced, such as singlet oxygen ($^1O_2$), which kill tumor cells. $^1O_2$ is a powerful, fairly indiscriminate oxidant that is generally recognized as the key agent of PDT-induced cell or virus damage. Because the diffusion range of $^1O_2$ is much smaller than the diameter of a single cell, the site of the primary generation of $^1O_2$ determines which subcellular structures may be attacked. Consequently, if a photosensitizer is preferentially localized in target cells, PDT-induced damage will be highly specific. A comprehensive review of porphyrins and their use as photosensitizers in photodynamic therapy appears in Pandey, R.K. and G. Zheng, "Porphyrins as Photosensitizers in Photodynamic Therapy" in *The Porphyrin Handbook*, Kadish, K.M. *et al.*, Eds., Academic Press (2000).

PDT has been used to sterilize blood because, although the interviewing of donors and serological screening have greatly reduced the contamination of blood by infectious agents, the risk of viral infections following blood transfusion still remains. Cytomegalovirus (CMV), hepatitis B (HBV) and C (HCV) viruses and human immunodeficiency virus (HIV) are the major causes of blood transfusion-transmitted diseases. To date, photodynamic treatment (PDT) has been one of the very few sterilization methods that may be applied to red blood cells (RBCs). When photosensitizer solutes are illuminated with light of the appropriate wavelength, reactive oxygen species (ROS) are formed, which have potent virucidal action.

Some extent of specificity is anticipated to be inherent in PDT as, in contrast to cells, viruses do not possess defense mechanisms against attack by ROS. For example, RBCs possess antioxidant defense systems that are enzymatic (methemoglobin reductase, superoxide dismutases, catalase, glutathione peroxidase) or nonenzymatic (endogenous scavengers such as reduced fluthathione and the vitamins A, C, and E) but, depending on the type and amount of ROS, these defense systems may fail. Virucidal phototreatment by use of methylene blue, for example, is associated with enhanced hemolysis, potassium leakage, and induction of binding of IgG and serum albumin to the RBC surface.

To prevent RBC damage, most of the research is based on three fronts: 1) to develop photosensitizer compounds that bind more selectively to viruses; 2) to impart additional protection to RBCs, mainly by inclusion of ROS scavengers, such as Trolox (a hydrophilic vitamin E derivative), glutathione, mannitol, and the RBC band III ligand dipyridamole; 3) to use long wavelength photosensitizers that have minimal light absorption by hemoglobin, such as chlorins and phthalocyanines.

In U.S. Patent No. 6,348,453, PDT has also been suggested as treatment to reduce HIV viremia in the blood of AIDS patients. Reduced HIV viremia in plasma is a predictor for enhanced survival of AIDS patients (Mellors, J.W., *et al.*, *Science* *272*:1167-1170 (1996). In addition, PDT agents have been used for treatment of early stage lung cancer, obstructive lung cancer, obstructive esophaegel cancer, high grade dysplasia in Barrett's esophagus, and other neoplasia. Treatment of lung cancer with PDT is recommended as a potentially curative treatment for microinvasive endobronchial cancer in patients who are not good candidates for or have refused surgery or radiation. A particularly new and important application is for the treatment of age-related macular degeneration (AMD), where PDT (Visudyne) has made a major impact on the outcome of this disease, the major cause of blindness in those over the age of 50. In the cancer field, while not yet approved (pending), the use of PDT in treatment of high-grade dysplasia (HGD) in Barrett's esophagus may well change how this disease is currently treated (often esophagectomy). Mechanistically, the recognition of apoptosis as an important mode of cell death following PDT and the critical role of the inflammatory process and immunity has only recently been recognized. Dougherty TJ, J Clin Laser Med Surg. 2002 Feb;20(1):3-7.

Fluorescence Resonance Energy Transfer

In 1990, Matayoshi *et al.* reported a fluorogenic substrate for assaying retroviral proteases (Matayoshi *et al.*, *Science 247*:954 1990) using the concept of fluorescence resonance energy transfer (FRET). This work used the quenched fluorogenic substrate (DABCYL as a quencher)-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln-(EDANS as a fluorophore; SEQ ID NO: 24), whose sequence is derived from a natural processing site for HIV protease, which is essential for the correct processing of viral polypeptides and the maturation of infectious virus. Thus, incubation of HIV protease with the fluorogenic substrate resulted in specific cleavage at the Tyr-Pro bond and a time-dependent increase in fluorescence intensity which was linearly related to the extent of substrate hydrolysis. This assay has greatly facilitated the development of HIV protease inhibitors for the control and treatment of AIDs. Since then, FRET-based enzyme-activated probes have been widely used in many other biological applications. The most notable one is the protease-activated near-infrared fluorescent probe concept developed by Ralph Weissleder and his colleagues for *in vivo* imaging of cancers.

If the FRET concept used in enzyme-activated fluorogenic substrates can be used to design novel PDT substrates, the PDT substrates would have a tremendous advantage over the current PDT agents because of greatly enhanced specificity. Greater specificity would ensure that there would be minimal, if any, damage to healthy tissues.

For example, HIV/AIDS is now the fourth biggest killer disease and was the root-cause of death for 2.2 million people in the year 1998. The situation is getting worse, especially in the developing world. Several anti-HIV drugs are now available on the market to control viral replication and to delay the onset of AIDS and death but, to date, no cure is available. At least four of the currently available drugs (Saquinavir, indinavir, nelfinavir & ritonavir) work by inhibiting the HIV viral aspartic proteinase that is responsible for processing the viral polyprotein. As a result, HIV proteinase has been extensively characterized in terms of its crystal structure and its substrate specificity. A key feature of this specificity is the ability of the enzyme to cleave N-terminal to a proline residue. Such cleavage is extremely rare and inhibitors designed from proline containing peptidomimetics have proved to be very selective for HIV proteinase (Roberts *et al.*, *Science* 248:358-361 (1990)). If the FRET concept used in enzyme-activated fluorogenic substrates can be used to design novel PDT substrates for HIV/AIDs, such PDT substrates would have a tremendous advantage over the current therapies.

BRIEF SUMMARY OF THE INVENTION

Recognizing the tremendous therapeutic potential of PDT substrates with enhanced specificity, the present inventors have developed substrates which can undergo a change of conformation in diseased (e.g., cancerous) and/or inflamed tissue.

Accordingly, in an aspect the invention is directed to a conjugate comprising a first substrate, at least one photosensitizer, and at least one first quencher, wherein the photosensitizer and the first quencher are each attached to said substrate, wherein the first substrate allows the photosensitizer and the first quencher to come sufficiently close to each other to facilitate quenching of an activated form of the photosensitizer.

In another aspect the invention is directed to a conjugate comprising a first substrate; a second substrate, the first substrate covalently linked to the second substrate; a first quencher, the first quencher attached to the first substrate; a second quencher, the second quencher attached to the second substrate and the second quencher comprising a fluorescence quencher; and a photosensitizer, the photosensitizer attached to the covalently linked first and second substrates and the photosensitizer comprising a fluorophore; wherein the first substrate allows the photosensitizer and the first quencher to come sufficiently close to each other to facilitate quenching of an activated form of the photosensitizer, and wherein the second substrate allows the photosensitizer and the second quencher to come sufficiently close to each other to facilitate quenching of fluorescence from the fluorophore of the photosensitizer.

In another aspect the invention is directed to a conjugate comprising a first substrate, wherein the first substrate is a nucleic acid, at least one photosensitizer, and at least one first quencher, the photosensitizer and the first quencher attached to the substrate, wherein the substrate allows the photosensitizer and the first quencher to come sufficiently close to each other to facilitate quenching of an activated form of the photosensitizer. In certain embodiments the nucleic acid comprises a first portion, a second portion, and a third portion, wherein the first portion and the third portion are at least about 70% complementary to each other. In other embodiments first portion and the third portion are at least about 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% complementary to each other. In the most preferred embodiment, the first portion and the third portion are 100% complementary to each other. In a preferred embodiment, the first portion and the third portion are capable of base pairing with each other resulting in a stem-loop structure wherein the first portion and the third portion form the stem and the second portion forms a non-base-paired loop region.

In another aspect, the invention is directed to a method of decontaminating blood comprising contacting blood with the conjugates of the present invention, and exposing the blood and substrate mixture to an effective amount of artificial irradiation.

In a further embodiment, the invention provides a method of treating a disease state contacting the diseased tissue with the conjugates of the present invention and exposing the diseased tissue to an effective amount of artificial irradiation.

BRIEF DESCRIPTION OF THE DRAWINGS/FIGURES

FIG. 1 Diagram depicting a conjugate which is an activatable PDT agent of the present invention, before and after cleavage by an enzyme. "PS" is the photosensitizer which generates reactive oxygen species (ROS), such as singlet oxygen ($^1O_2$) or superoxide free radicals; "Q" is the quencher which quenches the triplet state of the photosensitizer when the photosensitizer and quencher are in close proximity.

FIG. 2 Diagram of a conjugate which has an anti-sense nucleic acid as a first substrate.

FIG. 3 Diagram of a conjugate further comprising a death sensor.

FIG. 4 Diagram illustrating the categories of phospholipases, categorized as A1, A2, C and D based on their site of cleavage of a phospholipid.

FIG. 5 Synthesis of isothiocyanate-containing bacteriopurinimide, BChlPP-NCS.

FIG. 6 Chemical shifts of CH_2NHBOC, CH_2NH_2 and CN_2NCS in ^1H NMR spectra of functionalized bacteriochlorophylls.

FIG. 7 A) The HPLC chromatogram of BChlPP-NHS. RP-HPLC Column: ZARBOX-300SB_C8_4.6x250mm; Solvent A: 0.1% TFA, B: CH3CN; Gradient: From 10% B to 100% B for 45min; Flow: 1mL/min. At this condition, the retention time of BchlPP-2DG is 38.1min. Purity of the compound: >90%. B) Absorption (top) and Emission (bottom) spectra of BChlPP-NHS.

FIG. 8 Synthesis of BChlPP-NHS.

FIG. 9 A) The HPLC chromatogram of BChlE6 (Retention time:31.4min, purity:99%). B) The absorption spectrum of the 31.4min peak (BChlE6) obtained by HPLC. The maximum emission is 758nm(spectrum not shown).

FIG. 10 Absorption spectra of Pyro (a) and Car (b). (note: dotted line (left) indicating the emission spectrum of Pyro)

FIG. 11 Preparation of 7'-Apo-7'-(4-Carbomethoxyphenyl)-β-carotene.

FIG. 12 Preparation of Carotenide succinimide ester.

FIG. 13 The molecular structure of BChl-MBs.

FIG. 14 The structure of Pyro-30mer-Car.

FIG. 15 The synthesis of Pyro-30mer-Car.

FIG. 16 The absorption spectrum of Pyro-30mer (red line) and Pyro acid(Green line).

FIG. 17 The HPLC result (top) and the absorption spectrum (bottom)of Pyro-30mer-Car(A) and Pyro-30mer(B).

FIG. 18 The HPLC retention time and absorption spectrum of NH_2-30mer-Car.

FIG. 19 The HPLC result of the solution reaction (top) and the absorption spectrum of the peaks(bottom).

FIG. 20 The HPLC retention time and absorption spectrum of Pyro-30mer –Car

FIG. 21 The synthesis of Pyro –Peptide-Car(PPC)

FIG. 22 Figure 22.The HPLC retention time (top) and the UV-Visible spectrum of the HPLC peak (bottom) of Fmoc-peptide-NH_2. (Using RP C18 100A column and 0.1%TFA and CH_3CN as HPLC elution buffer, Gradient from 90% 0.1%TFA buffer to 100%CH_3CN in 45min).

FIG. 23 The HPLC retention time (the left) and the UV-Visible spectrum of the HPLC peak (the right) of Pyro-peptide. (Using RP C18 100A column and 0.1%TFA and CH_3CN as HPLC elution buffer, Gradient from 90% 0.1%TFA buffer to 100%CH_3CN in 45min).

FIG. 24 A) Structure of caspase-3 activatable Pyro-Peptide-Car. B) HPLC retention time of this construct and C) corresponding optical absorption spectrum. HPLC method: column: RP C8 300A; solvent:0.1%TFA, CH_3CN and MeOH; gradient: 60%(0.1%TFA) buffer and 40% CH_3CN to 10%(0.1%TFA) buffer and 90% CH_3CN in the first 20min, then to 90% CH_3CN and 10%MeOH for another 10min, finally to 80% CH_3CN and 20%MeOH for 10min).

FIG. 25 A) Fluorescence-based HPLC chromatograms (left column) and absorption spectra (right) corresponding to the dominant HPLC peaks for PPC alone (top), PPC+caspase-3 (middle) and PPC+caspase-3+inhibitor (bottom). B) Total $^1O_2$ luminescence counts for PP, PP + caspase-3, PP + caspase-3 + inhibitor, PPC, PPC + caspase-3, PPC + caspase-3 + inhibitor. C) Corresponding $^1O_2$ lifetime.

FIG. 26 a) Removing Fmoc, b) Labeling BChl, c) Cleaving CPG, d) Formation phosphoramidite, e) Synthesizing DNA from 3'-amino-modifier C7-CPG, f) Labeling Car at 3' end, g) Removing DMT, h) Linking ANT peptide via S-S bond.

FIG. 27 Synthesis of Pyro-GGHSSKLQGSGK-CAR beacon.

FIG. 28 Intracellular uptake of PPF (a model beacon) in $HepG_2$ tumor cells. A) 200μM PPF incubated with cells for 30 min; B) 200 μM PPF incubated with cells for 3 h; C) 200μM PPF incubated with cells for 24 h; D) cell alone control. Green color represents FITC fluorescence, red color represents Pyro fluorescence.

FIG. 29 Structure of the first enzyme-activatable PDT agent with a built in death sensor, BHQ-GDEVDSGK(Pyro)HSSKLQK-Car.

DETAILED DESCRIPTION OF THE INVENTION

Terms are used herein as generally used in the art, unless otherwise defined.

Conjugates

The present invention provides conjugates comprising at least one substrate. In an embodiment of the present invention, the PDT substrates of the present invention have following characteristics: 1) they contain a first substrate; 2) they contain a photosensitizer (P) and a first quencher (Q) attached to the substrate, wherein P and Q are held in proximity by the appropriate length of the substrate sequence. In preferred embodiments the first quencher is a triplet state quencher. Thus, Q quenches the triplet state of P and the subsequent formation of ROS is eliminated, therefore, PDT treatment will not harm the target cells and viruses. The first substrate is capable of changing conformation, and Q is removed from the immediate vicinity of P. Upon PDT treatment, P generates ROS to kill the target cells or viruses.

In certain embodiments, the change of conformation of the first substrate is caused by cleavage of the first substrate. Accordingly, in certain embodiments the first substrates of the conjugates of the present invention will be cleaved in the presence of specific enzymes. The specific enzymes will preferably be over-expressed, or present only, in diseased, e.g., virally infected, cancerous, and/or inflamed, tissue as compared to healthy tissues. Thus, upon exposure to light, the photosensitizer-containing cleavage products generate reactive oxygen species which preferentially destroy the diseased tissue. Because the photosensitizer is preferentially activated in target cells, damaged induced by the photosensitizer will be highly specific. Accordingly, preferred substrates are those that are cleavable by one or more cleavage enzymes.

First Substrates

As stated above, the conjugates of the present invention comprise at least one first substrate, one photosensitizer and one quencher. In general, the substrates of the present invention serve as a scaffold. In particular, when the substrate is intact, the quencher and the photosensitizer are held in proximity such that the quencher quenches the triplet state of the photosensitizer. Once the substrate is undergoes a conformation change, the quencher and the photosensitizer are no longer held in proximity and the photosensitizer is no longer quenched. Particularly preferred substrates are polypeptides, nucleic acid molecules, synthetic polymers, phospholipids, galactose-containing compounds, or combinations thereof. In certain embodiments the photosensitizer and first quencher are attached to the first substrate by a linker molecule.

In one embodiment of the present invention, the first substrate is a polypeptide which contains a site which is cleavable by a proteinase. As used herein, "proteinase" and "protease" are synonyms and refer to any enzyme that breaks down proteins by cleavage at one or more specific peptide bonds. Preferably, the first substrates of the present invention comprise one or more sites cleavable by a viral protease and/or an protease which is over-expressed, over-abundant or present only in, diseased tissue.

Generally, proteolytic enzymes cleave at specific amino acid residues. Therefore, in a preferred embodiment, the conjugates of the present invention comprise first substrates which contain specific residues that are recognized by viral proteases or proteases which are specific to, over-expressed in, or over-abundant in infected tissue. Preferred enzymes are proteases, preferably, viral proteases, and even more preferably, retroviral proteases.

In an embodiment, the present invention provides first substrates with the following structures: X-Tyr-Pro-Y, X-Lys-Lys-Y, X-Arg-Arg-Y, X-Gly-Ile-Y, X-Gly-Leu-Y, X-Ala-Ser-Y, X-Asp-Gly-Y, X-Phe-Phe-Y, X-Asp-Glu-Val-Asp(SEQ ID NO:1)-Y, X-Gly-Pro-Arg-Y, X-Arg-Gly-Y, or X-His-Ser-Ser-Lys-Leu-Gln(SEQ ID NO:2)-Y, wherein X and Y are each independently a polypeptide comprising from one to about 15 amino acids. Two designations for amino acids are used interchangeably throughout this application, as is common practice in the art: Alanine=Ala (A); Arginine=Arg (R); Aspartic Acid=Asp (D); Asparagine=Asn (N); Cysteine=Cys (C); Glutamic Acid=Glu (E); Glutamine=Gln (Q); Glycine=Gly (G); Histidine=His (H); Isoleucine=Ile (I); Leucine=Leu (L); Lysine=Lys (K); Methionine=Met (M); Phenylalanine=Phe (F); Proline=Pro (P); Serine=Ser (S); Threonine=Thr (T); Tryptophan=Trp (W); Tyrosine=Tyr (Y); Valine=Val (V).

In an embodiment of the present invention, when the photosensitizer is attached to X, a quencher is attached to Y; and when a quencher is attached to X, a photosensitizer is attached to Y. Preferably, there are at least from about 3 to about 10 first substrate amino acids between the photosensitizer and the quencher, more preferably there are from about 4 to about 8 first substrate amino acids between the photosensitizer and the quencher, and even more preferably, there are from about 5 to about 7 first substrate amino acids between the photosensitizer and the quencher.

In an aspect of the present invention, X and Y are each independently from 1 to about 25 amino acids, more preferably from 2 to about 15 amino acids, and even more preferably, from about 5 to about 10 amino acids in length.

In an additional embodiment, when the first substrate is a polypeptide, it is preferred that when the photosensitizer is attached to the N-terminal amino acid of the polypeptide, the quencher is attached to the C-terminal amino acid of the polypeptide; and when the photosensitizer is attached to the C-terminal amino acid of the polypeptide, the quencher is attached to the N-terminal amino acid of the polypeptide.

In an aspect of the invention, when the first substrate of the present invention is a polypeptide, and contains a protease-cleavable site, it is preferred that the protease-cleavable site is recognized by an aspartic proteinase. Aspartic proteinases are proteolytic enzymes which generally operate at acidic pH. Commonly an aspartic proteinase can accommodate about 7 residues of a first substrate in its active site cleft. These residues are usually designated as P4-P3-P2-P1*P1'-P2'-P3' with the scissile peptide bond between P1 and P1' indicated by "*". The corresponding subsites that constitute the topography of the active site cleft in each enzyme are designated accordingly as S4-S3-S2-S1-S1'-S2'-S3'. The scissile peptide bond between P1 and P1' residues normally consists of two hydrophobic residues although beta branched side chains such as valine or isoleucine are not favored in the P1 position. Generally, each lobe of an aspartic proteinase contributes one aspartic acid residue to the catalytic apparatus. These Asp residues are present in two ~Hydrophobic-Hydrophobic- Asp-Thr/Ser-Gly~ motifs. In pepsin the catalytic Asp residues are at positions 32 and 215. It is the essential role of these residues in coordinating a water molecule for nucleophilic attack on the scissile peptide bond which gives this class of enzyme its name.

An increasing number of aspartic proteinases are being characterized from vertebrates, insects, helminths, protozoans, plants, retroviruses and bacteria, and all such aspartic proteinases are encompassed within the definition of aspartic proteinase as used herein.

Preferred aspartic proteinases are rennin, chymosin and pepsin. A preferred pepsin is HIV-1 retropepsin.

HIV proteinase is a member of the aspartic proteinase family of enzymes. It is encoded by the virus and is essential to allow processing of the viral polyprotein. In contrast to the archetypal aspartic proteinases which are single chain enzymes, HIV proteinase is a homodimeric enzyme. Other retroviruses that infect vertebrates and plants produce aspartic proteinases which, like HIV proteinase, are symmetrical dimers.

Each monomer contributes an aspartic acid residue (Asp25) to the catalytic apparatus of the enzyme. As in the single chain aspartic proteinases, these Asp residues are found in ~Hydrophobic-Hydrophobic-Asp-Thr-Gly~ motifs.

Many diseased tissues (e.g., tumors) have been shown to have elevated levels of proteolytic enzymes, presumably in adaptation to rapid cell cycling and for secretion to sustain invasion, metastasis formation, and angiogenesis. In one aspect of the present invention, the conjugates of the present invention comprise substrates which have sites which are cleavable by such proteases. For example, in one embodiment the conjugates of the present invention are cleavable by viral enzymes. Such viral enzymes include, but are not limited to the following: HIV protease, cytomegalovirus protease, Ebstein-Barr virus protease, hepatitis B virus protease, hepatitis C virus protease, herpes simplex virus protease, cathepsin B, cathepsin D, a matrix metalloproteinase, cathepsin K, prostate-specific antigen, thrombin, caspase-3, and interleukin 1β converting enzyme. A preferred protease is human immunodeficiency virus type I protease.

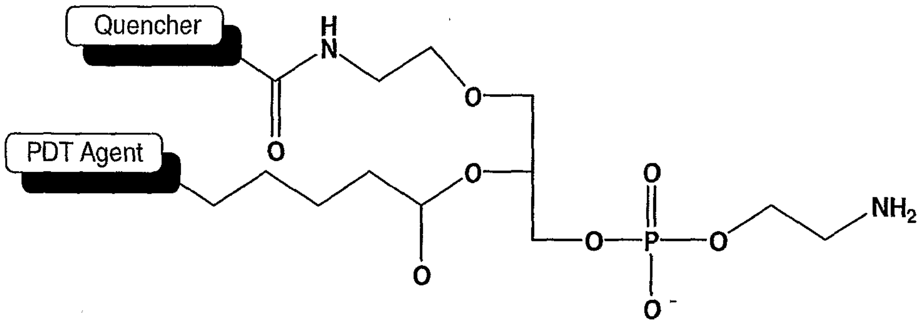
In an additional embodiment, the conjugates of the present invention comprise first substrates which are cleavable by thrombin, enzymes present during an inflammatory response, proteases (e.g., caspase 3 and 8), lipases (e.g., phospholipase A2), glycosidases (e.g., β-galactosidase), phosphatases (e.g., adenosine triphosphatase (ATPase), guanosine triphosphatase (GTPase), protein tyrosine phosphatase, deoxyribonuclease (DNAse), ribonuclease (RNAse), esterases (e.g., phosphodiesterase).

In an additional embodiment, the invention is directed to a conjugate which comprises a first substrate comprising a phospholipid which contains one or more sites which are cleavable by one or more phospholipase enzymes.

Phospholipases are enzymes that catalyze phospholipid breakdown. As illustrated in Figure 2, phospholipases are categorized as A1, A2, C and D based on their site of action. Phospholipase A1 and A2 (PLA1 and PLA2) remove fatty acid chains from the *sn*-1 and *sn*-2 positions of the glycerol backbone of a variety of phospholipids (Jackowski, S., *J. Biol. Chem.* *269*:3858 (1994)). Phospholipase C (PLC) specifically hydrolyzes the P-O bond adjacent to the glycerol *sn*-3 position to produce diacylglycerol and the corresponding phosphorylated head group. Phospholipase D (PLD) hydrolyzes the O-P bond adjacent to the head group, releasing the head group and a molecule of phosphatidic acid. Phospholipases are ubiquitous enzymes in plants and animals, and perform a number of critical regulatory functions. Phospholipases are involved in signal transduction, for the maintenance and turnover of membranes, as mediators or inflammation and immunity, and also act as digestive enzymes both at the cellular (i.e. lysosomal) level as well as being crucial to the absorption of nutrients through the gut.

Phospholipase A2 catalyzes the hydrolysis of the *sn*-2 bond of a phospholipid, creating a lysophospholipid and releasing a fatty acyl chain. Phospholipase A2 is critical in a number of functions at the cellular and tissue level as a modulator of inflammation, as an important regulator of immune function, as a controlling factor in signal transduction and in membrane re-modeling. PLA2 levels are increased during inflammatory response and in hyperproliferation. There are numerous isoforms of PLA2, which are generally divided into three categories on the basis of molecular weight and requirement for calcium. Among them, Type II PLA2 (sPLA2) is secreted by a number of cell types including eosinophils, mast cells and neutrophils. It is found in sera and inflammatory exudates of patients with chronic inflammatory diseases. sPLA2 has a specificity for phosphatidylethanolamine (PtdEtn) over phosphatidylcholine (PtdCho). The specificity for PtdEtn acts as a protection from self-hydrolysis, the outer leaflet of the plasma membrane is low in PtdEtn, whereas gram negative bacteria have membranes rich in PE. This enzyme can also act intracellularly for fatty acid turnover. sPLA2 levels are elevated in prostate cancer compared to normal prostate cells (Graff *et al.*, *Clinical Cancer Res. 7*:3857 (2001).

In an additional embodiment, the present invention is directed to a conjugate which comprises a first substrate having a site specific for Type II PLA2 (sPLA2). Preferably, the conjugate is a compound of Formula I:



I

wherein Q is a quencher and P is a photosensitizer.

In other embodiments the first substrate is a nucleic acid. Nucleic acids of the present invention can comprise any nucleotide, including adenine (A), cytosine (C), guanine (G), thymidine (T), and uracil (U) and analogs thereof. The backbone of the nucleic acids include but are not limited to a phosphodiester, a methyl phosphonate, a phosphorothioate, a borane phosphonate, a 3'-O-phosphopropylamino, a N3'-phosphoramidate, a 2'-O-alkyl-RNA, a morpholinophosphorodiamidate, and a peptide nucleic acid.

In a preferred embodiment the first substrate is a single stranded nucleic acid. In an especially preferred embodiment the nucleic acid comprises a first portion, a second portion, and a third portion, wherein the first portion and the third portion are at least 70% complementary to each other. Complementarity refers to Watson-Crick base pairing: adenine pairs with thymidine and uracil; guanine pairs with cytosine. In other embodiments first portion and the third portion are at least about 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% complementary to each other. In the most preferred embodiment, the first portion and the third portion are 100% complementary to each other. In a preferred embodiment the first portion and the third portion are capable of base pairing resulting in a stem-loop structure wherein the first portion and the third portion form the stem and the second portion forms a non-base-paired loop region. If the first quencher is attached to the first portion, then the photosensitizer is attached to the third portion. If the photosensitizer is attached to the first portion, then the first quencher is attached to the third portion. The first portion and the third portion need not be exactly the same length, but in preferred embodiments the first portion and the third portion are the same length. In a preferred embodiment the first portion and the third portion are both about 3 to about 10 nucleotides in length, preferably about 3 to about 7 nucleotides in length, and most preferably both the first portion and the third portion are about 5 nucleotides in length. In an especially preferred embodiment the first portion is SEQ ID NO: 3, 5'-gcgag-3' and the third portion is SEQ ID NO: 4, 5'-ctcgc-3'.

The second portion of the nucleic acid can comprise any nucleotide sequence. In certain embodiments the second portion is about 10 to about 50 nucleotides in length, preferably, about 10 to about 30 nucleotides in length, most preferably about 15 to about 25 nucleotides in length. In certain embodiments the second portion is a nucleic acid which is complementary to an mRNA molecule, preferably an mRNA which is overexpressed in a diseased cell, most preferably the mRNA is expressed at high levels in a cancer cell as compared to a normal cell. In certain embodiments the second portion is at least 70% identical to a nucleic acid sequence complementary to a nucleic acid sequence selected from the group consisting of c-Raf-1 mRNA, BRAF1 mRNA, DD3 mRNA, K-ras mRNA, CCND1 mRNA, and EGFRvIII mRNA. In other embodiments second portion is at least about 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% complementary to a nucleic acid sequence selected from the group consisting of c-Raf-1 mRNA, BRAF1 mRNA, DD3 mRNA, K-ras mRNA, CCND1 mRNA, and EGFRvIII mRNA. In the most preferred embodiment, the second portion is 100% complementary to a nucleic acid sequence selected from the group consisting of c-Raf-1 mRNA, BRAF1 mRNA, DD3 mRNA, K-ras mRNA, CCND1 mRNA, and EGFRvIII mRNA. In especially preferred embodiments the second portion is SEQ ID NO: 5, 5'-agctaggaaacaccaaagatgatatttg-3', or SEQ ID NO:6 5'-tcccgcctgtgacatgcatt-3'. Accordingly, preferred embodiments of the nucleic acid are SEQ ID NO:7, 5'-gcgagagctaggaaacaccaaagatgatatttgctcgc-3', and SEQ ID NO:8, 5'-gcgagtcccgcctgtgacatgcattctcgc-3'.

The present invention also is related to conjugates comprising a first substrate; a second substrate, the first substrate covalently linked to the second substrate; a first quencher, the first quencher attached to the first substrate; a second quencher, the second quencher attached to the second substrate and the second quencher comprising a fluorescence quencher; and a photosensitizer, the photosensitizer attached to the covalently linked first and second substrates and the photosensitizer comprising a fluorophore; wherein the first substrate allows the photosensitizer and the first quencher to come sufficiently close to each other to facilitate quenching of an activated form of the photosensitizer, and wherein the second substrate allows the photosensitizer and the second quencher to come sufficiently close to each other to facilitate quenching of fluorescence from the fluorophore of the photosensitizer. Accordingly, the photosensitizer can be attached to first substrate, the second substrate, or the junction of the first and second substrates.

The first substrate of this conjugate is as described above. Particularly preferred substrates are polypeptides, nucleic acid molecules, synthetic polymers, phospholipids, galactose-containing compounds, or combinations thereof. In certain embodiments the photosensitizer and second quencher are attached to the second substrate by a linker molecule.

Particularly preferred second substrates are polypeptides, nucleic acid molecules, synthetic polymers, phospholipids, galactose-containing compounds, or combinations thereof. In certain embodiments the second substrate is cleavable. In a preferred embodiment the second substrate is a polypeptide. In a preferred embodiment the second substrate is cleavable by a caspase enzyme. In a particularly preferred embodiment, the second substrate is cleavable by a protease selected from the group consisting of caspase-1, caspase-2, caspase-3, caspase-4, caspase-5, caspase-6, caspase-7, caspase-8, caspase-9, and caspase-10. In an especially preferred embodiment, the protease is caspase-1, caspase-3, or caspase-9.

In a preferred embodiment, the first substrate and the second substrate are polypeptides, and the C- terminal amino acid of the first substrate and the N-terminal amino acid of the second substrate are covalently linked by a peptide bond. In an especially preferred embodiment, the photosensitizer is attached to either the C-terminal amino acid of the first substrate or the N-terminal amino acid of the second substrate, and the first quencher is attached to the N-terminal amino acid of the first substrate; and the second quencher is attached to the C-terminal amino acid of the second substrate.

In an alternative preferred embodiment, the first substrate and the second substrate are polypeptides, and the C-terminal amino acid of the second substrate and the N- terminal amino acid of the first substrate are covalently linked by a peptide bond. In an especially preferred embodiment, the photosensitizer is attached to the C-terminal amino acid of the second substrate or the N-terminal amino acid of the first substrate, and the first quencher is attached to the C-terminal amino acid of the first substrate; and the second quencher is attached to the N-terminal amino acid of the second substrate.

In a preferred embodiment the second substrate comprises a sequence selected from the group consisting of Asp-Glu-Val-Ile(SEQ ID NO: 9), Asp-Glu-Thr-Asp(SEQ ID NO: 10), Leu-Glu-His-Asp(SEQ ID NO: 11), Asp-Glu-His-Asp(SEQ ID NO: 12), Trp-Glu-His-Asp(SEQ ID NO: 13), Leu-Glu-Thr-Asp(SEQ ID NO: 14), Asp-Glu-Val-Asp(SEQ ID NO: 15), Val-Glu-His-Asp(SEQ ID NO: 16), and Ile-Glu-Ala-Asp(SEQ ID NO: 17).

In an alternative preferred embodiment, the second substrate comprises a sequence selected from the group consisting of X-Asp-Glu-Val-Ile(SEQ ID

NO: 9)-Y, X-Asp-Glu-Thr-Asp(SEQ ID NO: 10)-Y, X-Leu-Glu-His-Asp(SEQ ID NO: 11)-Y, X-Asp-Glu-His-Asp(SEQ ID NO: 12)-Y, X-Trp-Glu-His-Asp(SEQ ID NO: 13)-Y, X-Leu-Glu-Thr-Asp(SEQ ID NO: 14)-Y, X-Asp-Glu-Val-Asp(SEQ ID NO: 15)-Y, X-Val-Glu-His-Asp(SEQ ID NO: 16)-Y, and X-Ile-Glu-Ala-Asp(SEQ ID NO: 17)-Y, wherein X and Y are each independently a polypeptide comprising from one to about 15 amino acids and the N-terminal amino acid of X is covalently linked to the first substrate.

In an alternative preferred embodiment, the second substrate comprises a sequence selected from the group consisting of X-Asp-Glu-Val-Ile(SEQ ID NO: 9)-Y, X-Asp-Glu-Thr-Asp(SEQ ID NO: 10)-Y, X-Leu-Glu-His-Asp(SEQ ID NO: 11)-Y, X-Asp-Glu-His-Asp(SEQ ID NO: 12)-Y, X-Trp-Glu-His-Asp(SEQ ID NO: 13)-Y, X-Leu-Glu-Thr-Asp(SEQ ID NO: 14)-Y, X-Asp-Glu-Val-Asp(SEQ ID NO: 15)-Y, X-Val-Glu-His-Asp(SEQ ID NO: 16)-Y, and X-Ile-Glu-Ala-Asp(SEQ ID NO: 17)-Y, wherein X and Y are each independently a polypeptide comprising from one to about 15 amino acids and the C-terminal amino acid of Y is covalently linked to the first substrate.

In an especially preferred embodiment the conjugate comprises pyropheophorbide (Pyro) as a photosensitizer, a carotenoid (Car) as a first quencher (Q_s), a black hole quencher (BHQ) as a second quencher (Q_f), with the second substrate between Pyro and Q_s comprising a caspase-3 substrate (GDEVDGSGK) (SEQ ID NO:18).

Photosensitizer

As discussed previously, the conjugates of the present invention comprise a first substrate, a first quencher and a photosensitizer. As used herein, the term "photosensitizer" encompasses any agent used in photodynamic therapy. If not quenched, such agents become activated upon exposure to light and oxygen, producing lethal reactive oxygen species that kill, for example, tumor cells. Accordingly, an activated form of a photosensitizer produces lethal reactive oxygen species. In a one embodiment, the photosensitizers used in the present invention generate singlet oxygen upon exposure to oxygen and light of the appropriate wavelength.

In an aspect of the present invention, the photosensitizer is a free base or metal complex of a compound selected from the group consisting of a porphyrin (e.g., porphyrin), a reduced porphyrin (e.g., chlorin), a chlorophyll, a chlorophyll derivative (e.g., phyropheophorbide, chlorin e6, chlorin p6 and purpurin 18), synthetic chlorin (e.g., a benzoporphyrin derivative and purpurin), bacteriochlorin (e.g., bacteriochlorophyll derivative, synthetic bacteriochlorin, porphyrin isomer (e.g., porphycence, heteroatom-fused porphyrin and inverted porphyrin), an expanded porphyrin (e.g., texaphyrin), and porphyrin analog (e.g., phthalocyanine and naphthalocyanine). In addition, the photosensitizer can be a nonporphyrin (e.g., hypericin, cationic dye (i.e., rhodamine), psoralen, and merocyanine 540).

Additional photosensitizers for use in the conjugates of the present invention will be apparent to one of skill in the art. As stated above, preferred photosensitizers are those used in photodynamic therapy. Even more preferred photosensitizers are those that have undergone or are currently undergoing clinical trials. For example, photosensitizers listed in Pandey, R.K. and G. Zheng, "Porphyrins as Photosensitizers in Photodynamic Therapy" in *The Porphyrin Handbook*, Kadish, K.M. *et al.*, Eds., Academic Press (2000), which is hereby incorporated by reference in its entirety, can be used in the conjugates of the present invention.

Quenchers

As used herein, the term "first quencher" encompasses any agent which quenches the triplet state of the photosensitizer, so that no photoreaction occurs upon exposure to light and oxygen, and no ROS is generated.

In a preferred embodiment the second quencher is a non-fluorescent chromophore that overlaps with the photosensitizer's emission (a black hole quencher). In a particularly preferred embodiment the second quencher is DABCYL (4-(4'-dimethylaminophenylazo) benzoic acid). Other second quenchers include "BHQ-0", "BHQ-1", "BHQ-2" and "BHQ-3" and commercially available from Biosearch Technologies, Inc., Novato, CA.

As used herein, the term "second quencher" encompasses any agent which quenches fluorescence of the photosensitizer, so that no photoreaction occurs upon exposure to light and oxygen, and no ROS is generated. In a separate embodiment of the present invention, the second quencher used in the conjugates of the present invention is a carotenoid, a metal complex dye, a cyanine dye, a stilbene quinone dye, an azomethine dye, an amine, a phenol, a sulfide, a bilirubin, a biliverdin, a nitroso compound, a nitrone compound or a N-oxy compound.

Methods of Treatment

The present invention is directed to a method of inhibiting the growth of cancer cells, *in vitro* or *in vivo*, comprising contacting the cancer cells with a conjugate of the present invention and exposing the cancer cells to an effective amount of artificial irradiation. In one aspect, the invention provides methods of inhibiting the growth of cancer cells, such as breast, lung, pancreas, bladder, ovarian, testicular, prostate, retinoblastoma, Wilm's tumor, adrenocarcinoma or melonoma, and preferably, the cancer tumor is a prostate cancer tumor.

The present invention is also directed to a method of inhibiting plaque formation in blood vessels comprising contacting a subject's blood and/or blood vessels with a conjugate of the present invention and exposing the blood and/or blood vessels to an effective amount of artificial irradiation.

The present invention is further directed to a method of decontaminating blood comprising contacting blood with the conjugates of the present invention and exposing the blood and conjugate mixture to an effective amount of artificial irradiation. As used herein, "decontaminating" means that the level of infectious virus is reduced in such a manner that the majority of all of the infectious virus contained in the blood is destroyed or inactivated. In a preferred embodiment, 60%, 70%, 80%, 90%, 95%, 96%, 97%, 98% or 99% of the virus in the blood is destroyed or inactivated. The present invention provides blood decontamination methods that inactivate or reduce the viral count in the blood by about 1 to about 20 log, and preferably by about 2 to about 15 log, and most preferably by about 4 to about 6 log.

The term " blood" as used herein encompasses whole blood, and fractions of whole blood, such as plasma. The term "contacting" as used herein encompasses mixing or stirring the conjugates of the present invention, which may be present in a pharmaceutically acceptable carrier. A carrier is deemed "pharmaceutically acceptable" if it is compatible with the other ingredients of the formulation and is not deleterious. For example, the conjugates of the present invention may be combined with a sterile aqueous solution which is preferably isotonic with the virus containing blood. Such formulations may be prepared by combining the conjugate formulation with water containing physiologically compatible substances such as sodium chloride, glycine, and the like, and having a buffered pH compatible with physiological conditions to produce an aqueous solution, and rendering said solution sterile. The formulations may be present in unit or multi-dose containers such as sealed ampoules or vials.

In an embodiment of the present invention, the light is applied immediately following contact between the conjugates of the present invention and the blood. In a further embodiment of the present invention, the light is applied from about 1 minute to about 3 hours following contact between the conjugates of the present invention and the blood, and preferably about 5 to about 60 minutes following contact between the conjugates of the present invention and the blood. The light should be applied at a sufficient wavelength, dose and duration to maximize the inactivation of infectious virus, and at the same time, to minimize the damage to the red blood cells and/or other surrounding tissue.

The specific dose and duration of light again will depend upon the photosensitizer chosen and/or the contamination of the blood. The dose of light applied is preferably about 5-25 mW/cm^2, and most preferably about 18-22 mW/cm^2, while the duration of light application is about 5-60 minutes, and most preferably about 20-30 minutes. Suitable sources of light include commercially available lasers, lamps, light emitting diodes and the like.

Preferably, LED arrays (Efos Canada, Inc., Mississauga, Ontario, Canada) are employed. To achieve the desired wavelength of light, the lamp may be equipped with commercially available filters.

Various photosensitizers for use in the present invention are useful over the range of 350 to 1300 nm, the exact range being dependent upon the particular photosensitizer. Preferred photosensitizers are those useful in the range of 650-1000 nm (i.e., in the near infrared ("NIR")). For example, pyropheophorbide is useful in the 650-900 nm range.

It also is within the confines of the present invention that one or more quenchers can be administered before, during or after the administration of the conjugates of the present invention, but before application of light. Suitable quenchers include but are not limited to glutathione, trolox, flavonoids, vitamin C, vitamin E, cysteine and ergothioneine and other non-toxic quenchers, and preferably vitamin E. The amount of the quencher administered will depend upon the specific quencher(s) chosen and can be determined by one skilled in the art. However, when the quencher is vitamin E, the preferred dose ranges from about 10 mg/kg body weight to about 1 g/kg body weight, and most preferably about 100 mg/kg body weight. Administering one or more of the aforementioned quenchers is optional, and is complimentary to administering the conjugates of the present invention. Preferred complementary quenchers, such as vitamin E, quench free radical formation generated from a Type I photoreaction via electron transfer, and may be used as a complementary protection mechanism to quenching singlet oxygen that is generated from a Type II photoreaction via energy transfer. Nevertheless, singlet oxygen is the major cytotoxic agent responsible for PDT.

In one embodiment of the present invention, the artificial irradiation is applied from about 5 minutes to about 3 hours after administering one or more types of conjugates of the present invention. Preferably, the artificial irradiation is applied about 10-60 minutes after administering one or more kinds of conjugates of the present invention.

The light should be applied at a sufficient wavelength, dose and duration to maximize the inactivation of infectious virus, and at the same time, to minimize the damage to the red blood cells and/or other surrounding tissue.

The specific dose and duration of light again will depend upon the photosensitizer chosen. The dose of light applied is preferably about 5 to about 25 mW/cm^2, and most preferably about 18 to about 22 mW/cm^2, while the duration of light application is about 5 to about 60 minutes, and most preferably about 20 to about 30 minutes. Suitable sources of light include commercially available lasers, lamps, light emitting diodes and the like. Preferably, LED arrays (Efos Canada, Inc., Mississauga, Ontario, Canada) are employed. To achieve the desired wavelength of light, the lamp may be equipped with commercially available filters.

In a separate embodiment, the invention is directed to a method wherein the artificial irradiation is applied for about 5 seconds to about 60 minutes, and preferably it is applied for about 1 minute to about 45 minutes, and even more preferably, artificial irradiation is applied for about 10 to about 30 minutes.

In a preferred embodiment, in the methods of the present invention, the artificial irradiation is selected from the group consisting of artificial ultraviolet, infrared (IR), gamma-irradiation, x-ray and visible light. Preferably, the artificial irradiation is IR, and even more preferably, the artificial irradiation is near-infrared (NIR).

In a preferred embodiment, the artificial irradiation is applied at a wavelength ranging from about 20 nm less than the maximum absorption of the photosensitizer to about 20 nm greater than the maximum absorption of the photosensitizer.

In the methods of the present invention, it is preferred that the artificial irradiation is applied about 30 minutes to about 48 hours after administering the conjugate of the present invention (e.g., by injection), and even more preferably, about 3 to about 24 hours after administering the conjugate of the present invention. Preferably, the light dose is 10 mW/cm^2 to about 150 mW/cm^2, more preferably about 35 to about 100 mW/cm^2, and most preferably about 75 mW/cm^2.

In a separate embodiment, in the methods of the present invention, it is preferred that the artificial irradiation be applied for about 5 seconds to about 60 minutes, even more preferred is that the artificial irradiation be applied for about 1 minute to about 45 minutes, and most preferably, in the methods of the present invention, the irradiation is applied for about 10 to about 30 minutes.

The present invention provides a method for selectively killing tumor cells expressing an enzyme that specifically cleaves the substrate of the present invention or its functional equivalent. For example, this invention provides a method of treating carcinomas (for example human carcinomas) *in vivo*. This method comprises administering to a subject a pharmaceutically effective amount of a composition containing at least one of the conjugates of the present invention.

In accordance with the practice of this invention, the subject may be a human, equine, porcine, bovine, murine, canine, feline, and avian subjects. Other warm blooded animals are also included in this invention.

The present invention also provides a method for curing a subject suffering from a cancer. The subject may be a human, dog, cat, mouse, rat, rabbit, horse, goat, sheep, cow, chicken. The cancer may be identified as a breast, lung, pancreas, bladder, ovarian, testicular, prostate, retinoblastoma, Wilm's tumor, adrenocarcinoma or melonoma and is generally characterized as a group of cells which over-express and/or have an over-abundance of specific cleavage enzymes. This method comprises administering to the subject a cancer killing amount of one or more conjugates of the present invention.

Also provided is a method of inhibiting the proliferation of mammalian tumor cells which comprises contacting the mammalian tumor cells with a sufficient concentration of the conjugate of the invention, followed by exposure to artificial irradiation, so as to inhibit proliferation of the mammalian tumor cells.

The subject invention further provides methods for inhibiting the growth of human tumor cells, treating a tumor in a subject, and treating a proliferative type disease in a subject. These methods comprise administering to the subject an effective amount of the conjugate of the invention.

The present invention also provides for a method of treating a disease state comprising administering to a target tissue of a patient a conjugate of the present invention and irradiating the photosensitizer thereby killing the target tissue.

The present invention also provides for a method of treating a disease state comprising administering to a target tissue of a patient a conjugate of the present invention and irradiating the photosensitizer thereby killing the target tissue, wherein a second substrate is cleaved by a protease which removes a second quencher from a conjugate and allows fluorescence from a fluorophore of the photosensitizer to be detected.

The present invention also provides for a method of treating a disease state comprising

(a) administering to a target tissue of a patient a conjugate comprising:

a first substrate comprising a nucleic acid wherein the nucleic acid comprises a first portion, a second portion, and a third portion, the first portion and the third portion capable of base-pairing resulting in a stem-loop structure wherein the first portion and the third portion form the stem and the second portion forms a non-base-paired loop region,

at least one photosensitizer, and

at least one first quencher, the photosensitizer and the first quencher attached to the first substrate, said first substrate capable of bringing said photosensitizer and the first quencher sufficiently close to each other to facilitate quenching of an activated form of the photosensitizer wherein said first substrate undergoes a change of conformation in the target tissue such that formation of the stem-loop structure is inhibited, and

(b) irradiating the photosensitizer thereby killing the target tissue.

In a preferred embodiment, the change of conformation of the first substrate is facilitated by the annealing of the second portion to a nucleic acid present in the target tissue.

It is apparent therefore that the present invention encompasses pharmaceutical compositions, combinations and methods for treating human carcinomas. For example, the invention includes pharmaceutical compositions for use in the treatment of human carcinomas comprising a pharmaceutically effective amount of the conjugate of the present invention and a pharmaceutically acceptable carrier.

The compositions may additionally include other drugs or antibodies treating carcinomas.

The conjugates of the invention can be administered using conventional modes of administration including, but not limited to, intravenous, intraperitoneal, oral, intralymphatic or administration directly into the tumor. Intravenous administration is preferred.

The compositions of the invention may be in a variety of dosage forms which include, but are not limited to, liquid solutions or suspension, tablets, pills, powders, suppositories, polymeric microcapsules or microvesicles, liposomes, and injectable or infusible solutions as well as conjugates of the above with polyethylene glycol (pegylated carriers). The preferred form depends upon the mode of administration and the therapeutic application.

The compositions of the invention also preferably include conventional pharmaceutically acceptable carriers and adjuvants known in the art such as human serum albumin, ion exchangers, alumina, lecithin, buffer substances such as phosphates, glycine, sorbic acid, potassium sorbate, and salts or electrolytes such as protamine sulfate.

The most effective mode of administration and dosage regimen for the compositions of this invention depends upon the severity and course of the disease, the patient's health and response to treatment and the judgment of the treating physician. Accordingly, the dosages of the compositions should be titrated to the individual patient. Nevertheless, an effective dose of the compositions of this invention may be in the range of from about 1 to about 2000 mg/kg. Preferably, the dosage is from about 2 to about 1000 mg/kg, more preferably, 4 to about 400 mg/kg, and even more preferably, 5 to about 100 mg/kg.

The conjugates described herein may be in a variety of dosage forms which include, but are not limited to, liquid solutions or suspensions, tablets, pills, powders, suppositories, polymeric microcapsules or microvesicles, liposomes, and injectable or infusible solutions as well as conjugates of the above with polyethylene glycol (pegylated carriers). The preferred form depends upon the mode of administration and the therapeutic application.

The most effective mode of administration and dosage regimen for the conjugates of the present invention depends upon the location of the tumor being treated, the severity and course of the cancer, the subject's health and response to treatment and the judgment of the treating physician. Accordingly, the dosages of the conjugates should be titrated to the individual subject.

The interrelationship of dosages for animals of various sizes and species and humans based on mg/kg of surface area is described by Freireich, E. J. *et al.*, *Cancer Chemother. 50 (4)*: 219-244 (1966). Adjustments in the dosage regimen may be made to optimize the tumor cell growth inhibiting and killing response, e.g., doses may be divided and administered on a daily basis or the dose reduced proportionally depending upon the situation (e.g., several divided doses may be administered daily or proportionally reduced depending on the specific therapeutic situation.

The dose of the composition of the invention required to achieve cures may be further reduced with schedule optimization.

In accordance with the practice of the invention, the pharmaceutical carrier may be a lipid carrier or lipoprotein particle such as LDL, HDL, VLDL, IDL or chylomicron. The lipid carrier may be a phospholipid. Further, the lipid carrier may be a fatty acid. Also, the lipid carrier may be a detergent. As used herein, a detergent is any substance that alters the surface tension of a liquid, generally lowering it.

In one example of the invention, the detergent may be a nonionic detergent. Examples of nonionic detergents include, but are not limited to, polysorbate 80 (also known as Tween 80 or (polyoxyethylenesorbitan monooleate), Brij, and Triton (for example Triton WR-1339 and Triton A-20).

Alternatively, the detergent may be an ionic detergent. An example of an ionic detergent includes, but is not limited to, alkyltrimethylammonium bromide.

Additionally, in accordance with the invention, the lipid carrier may be a liposome or polymerosome as well as conjugates of the above with polyethylene glycol (pegylated carriers). As used in this application, a "liposome" is any membrane bound vesicle which contains any molecules of the invention or combinations thereof.

In an additionally preferred embodiment, the present invention is directed to a method of decontaminating blood in a subject comprising administering to the subject the conjugates of the present invention; and exposing said subject to an effective amount of artificial irradiation. Preferred subjects are mammals and preferred mammals are humans.

The human is preferentially exposed to artificial irradiation is selected from the group consisting of artificial ultraviolet, infrared (IR), gamma-irradiation, x-ray and visible light. Preferred irradiation is IR, and even more preferably, the IR is near-infrared (NIR). Preferably, the artificial irradiation is applied about 5 minutes to about 3 hours after administering the of the present invention, even more preferably, the artificial irradiation is applied about 10 to about 60 minutes after administering the conjugate of the present invention.

The amount of conjugate administered in the formulation will depend upon the photosensitizer chosen. Preferably, the amount of conjugate administered is about 0.1 to about 10.0 mg/kg body weight of the subject, more preferably about 0.3 to about 6 mg/kg body weight, and even more preferably, about 0.4 to about 4.0 mg/kg body weight.

In a preferred embodiment, in the methods of treating cancer of the present invention, the artificial irradiation is applied for about 10 seconds to about 60 minutes, and even more preferably, the artificial irradiation is applied for about 15 seconds to about 30 minutes.

In a separate embodiment, the present invention further provides pharmaceutical compositions which comprise the conjugates of the present invention and a pharmaceutically acceptable carrier.

The present invention further provides a method of treating cancer in a subject cancer comprising administering a therapeutically effective amount of the pharmaceutical composition of the present invention.

The present invention further provides a method of treating viral infections in a subject, comprising administering a therapeutically effective amount of the pharmaceutical composition of the present invention.

The examples below explain the invention in more detail. The following preparations and examples are given to enable those skilled in the art to more clearly understand and to practice the present invention. The present invention, however, is not limited in scope by the exemplified embodiments, which are intended as illustrations of single aspects of the invention only, and methods which are functionally equivalent are within the scope of the invention. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

EXAMPLES

Example 1—Preparation of a Conjugate

Synthesis of Photosensitizers--Stable Bacteriochlorophyll Analogs (BChl):

Bacteriochlorophyll (BChl) from *R. Sphaeroides* is an excellent near-infrared (NIR) dye for NIR imaging and photodynamic therapy (PDT), but it is unstable. This example describes efficient synthesis of isothiocyanate-containing BChl analogs derived from bacteriopurpurinimide (BChlPP) and bacteriochlorin e_6 (BChlE6). Introducing an amine reactive universal linker such as isothiocyanate or succinimide ester into the BChl macrocycle allows conjugation of these NIR dyes to oligonucleotide or peptide by coupling of the terminal primary amine group.

Synthesis of Bacteriopurpurin-18-N-3′-(isothiocyanate)propylimide, BChlPP-NCS

Bacteriopurpurin-18-N-3′-(amino)propylimide (BChlPP-NH_2, was synthesized from bacteriopurpurin-18-N-3′-(BOC-amino)propylimide (BChlPP-BOC) and converted successfully to bacteriopurpurin-18-N-3′-(isothiocyanate)propylimide (BChlPP-NCS) as shown in Figure 5 (Chen, Y., et al., J Med Chem. 2002 Jan 17;45(2):255-8). However, the overall yield of BChlPP-NCS from BChlPP is just 10% with BChlPP-BOC formation as the yield-limiting step. The conversion of BChlPP to its corresponding BChlPP-BOC, BChlPP-NH_2 and BChlPP-NCS was clearly demonstrated by their NMR spectra. As shown in Figure 5, the 5.62 and the 3.31 ppm peaks observed in the BChlPP-BOC spectrum (shown partially) belonged to the N-H proton and the CH_2N protons adjacent to the BOC protection group, respectively. Cleavage of the BOC group led to the disappearance of the N-$\underline{H}$ peak and the upfield shift of the $C\underline{H_2}N$ resonance to 2.95 ppm. Further conversion of BChlPP-NH_2 to BChlPP-NCS shifts the above mentioned $C\underline{H_2}$ resonance downfield to 3.85 ppm. Figure 6 shows the HPLC chromatogram and the absorption spectra of this compound.

Synthesis of Photosensitizers--Bacteriochlorin e_6-13-carboxy-N-3'-(isothiocyanate)propylamide (BChlE6-NCS)

To improve the feasibility of using BChl-based bioconjugates for cancer detection and treatment, another synthetic strategy to functionalize BChl was employed. Bacteriopheophorbide *a* methyl ester was first reacted with *tert*-butyl N-(-3-aminopropyl)-carbamate to form a single regioisomer, bacteriochlorin e_6-13-carboxy-N-3'-(BOC-amino)propylamide, BChlE6-BOC. This intermediate was then converted to its corresponding amino- and isothiocyanate-containing BChl (BChlE6-NH_2 and BChlE6-NCS) as described above. Compared with the previous procedure (10% overall yield from BChlPP to BChlPP-NCS, see Figure 5), the new synthetic route from bacteriopheophorbide *a* methyl ester to BChlE6-NCS shown in Figure 8 has the overall yield of 45%. Considering that bacteriopheophorbide *a* methyl ester is the precursor of BChlPP, the actual yield improved is five- to ten-fold. The purity of BChlE6-NHS was 99% by RP-HPLC. Figure 8 shows the HPLC chromatogram and the absorption spectra of this compound.

Synthesis of Photosensitizers-- BChl, Pyrophephorbide acid and Pyrophephorbide succinimide ester synthesis

Bacteriochlorophyll (BChl) is known as an excellent photosensitizer for NIR imaging and photodynamic therapy (PDT), but it is unstable and expensive. In order to establish a optimal protocol for synthesis of the BChl-Car-MB, an inexpensive starting material, pyropheophorbide acid(Pyro), which was prepared from chlorophyll (Chl, the plant counterpart of BChl) extracted from *Spirulina* algae was selected as PS for synthesis of PDT beacon. Pyropheophorbide acid has a distinctive Soret band (380 - 420 nm) and a Qy band at 664 nm (see Figure 10a (Solid line). It also has strong emission at 672nm and good photophysical properties ($^1O_2$ yield: 45%) . Figure 10b showed the absorption spectrum of carotenoid (Car) moieties

Methyl pheophorbide a (500 mg, 0.82mmol) isolated from Spirulina algae was heated under refluxed temperature in collidine (100mL) for 3 h under nitrogen atmosphere. The solution was evaporated under high vacuum, and the residue so obtained was chromatographed over an alumina column (Grade III) and eluted with CH_2Cl_2. Pyropheophorbide a methyl ester was crystallized from CH_2Cl_2/hexane in 91% yield (411 mg, 0.75 mmol). This intermediate (250 mg, 0.46 mmol) was dissolved in THF (65mL) and mixed with a solution containing LiOH (500 mg), methanol (7 mL), and water (3 mL). The mixture was stirred under argon atmosphere for 24 h. After workup, the crude product was chromatographed on a silica column with 10% methanol in CH_2Cl_2 to give the title compound in 82% yield (200 mg, 0.37 mmol). The spectral and physical data of compound are consistent with the literature. Mp: 220-223°C. UV-vis in CH_2Cl_2: 411 nm (ε 1.1 x 10^5), 509 (1.1 x 10^4), 537(9.6 x 10^3), 611(8.2 x 10^3), and 669(4.5 x 10^4). Mass calcd for $C_{33}H_{34}N_4O_3$: 534.5; found by ESI-MS; 535.6(MH^+_) and 557.6($M+Na^+$). ^1H NMR ($CDCl_3$): 9.47, 9.35 and 8.53 (each s, 1H, 5-H, 10-H, and 20-H); 8.00(dd, *J*=17.7, 11.4 Hz, 1H, 3^1-$CH=CH_2$); 6.27(d, *J*=17.7Hz, 1H, *trans*-3^2-$CH=CH_2$); 6.15(d, *J*=11.4 Hz, 1H, cis-3^2-$CH=CH_2$); 5.18(ABX, 2H, 13^2-CH_2); 4.47(q, *J*=7.1, 1.9 Hz, 1H for 18-H); 4.29(m, *J*=7.8 Hz, 1H for 17-H); 3.68(q, *J*=7.4 Hz, 2H, 8-CH_2CH_3); 3.64, 3.39,and 3.22 (each s, 3H, 12-CH_3, 2-CH_3 and 7-CH_3); 2.65 and 2.32 (each m, 2H, for 2 x 17^1-H and 2 x 17^2-H); 1.81 (d, J=7.2 Hz, 3H, 18-CH_3); 1.70 (t, J=8.3 Hz, 3H, 8-CH_2CH_3); 0.87 and -1.35 (each brs, 1H, 2 x N-H).

Synthesis of Quenchers--Synthesis of Carotenoid Precursors (Car):

The following is a description of efficient synthesis of succinimide ester-containing Carotenoid Precursors(Car), which allows conjugation of Car to oligonucleotide or peptide by coupling of the terminal primary amine group.

Synthesis of Quenchers--Preparation of diethyl (4-carbomethoxy)benzylphosphonate

Methyl-4(bromomethyl)benzoate (5.00g, 21.8 mmol) and triethyl phosphate (6.51g, 39.2 mmol) in toluene (50 mL) were stirred under a stream of argon. The mixture was heated at reflux for 28 h. The solvent was distilled under reduced pressure and the residue was purified by flash chromatography (dichloromethane/gradient methanol, 5%) to afford 5.87 g (94 %) of pure phosphonate as determined by NMR spectroscopy. UV λ_{max} (dichloromethane) [nm] (ε [$dm^3mol^{-1}cm^{-1}$]) 236 (1.75×10^4), 270 (1.28×10^3). ^{1}HNMR ($CDCl_3$, TMS) δ 1.23 (t, 6H, J=7.2 Hz); 3.19 (d, 2H, J=21.9 Hz); 3.90 (s, 3H); 4.02 (q, 4H, J=7.2 Hz); 7.36 (d, 2H, J=8.1 Hz); 7.98 (d, 2H, J=8.1 Hz). ^{13}CNMR (75.45 MHz, $CDCl_3$, TMS) δ 16.31 (d, Jpocc=5.4 Hz, -CH_3); 34.00 (d, Jpc=138.5, -$OPCH_2$-); 52.06 (-OCH_3); 62.52 (d, Jpoc=7.6 Hz, -CH_2OPO-); 128.78 ($1C_{Ar}$); 129.75 ($2CH_{Ar}$); 129.84 (d, $J_{PCH2CArCAr}$=6.4 Hz, $2CH_{Ar}$); 137.17 (d, $J_{PCH2CAr}$=8.6 Hz, $1CH_{Ar}$); 166.88 (-$COOCH_3$). MS [m/z] 286.1 (M^+).

Into a 250 mL flask outfitted with a magnetic stirring bar, a condenser, and a gas inlet tube are placed 1.0 g(2.4 mmol) of 8'-apo-β-carotenal, 50 mL of THF 1g (2.9 mmol) of diethyl(4-carbomethoxy)benzylphosphonate and 0.17 g (3.1 mmol) of sodium methoxide. The suspension was stirred for overnight at room temperature. The crude mixture was neutralized with hydrochloric acid. The solution poured with 500 mL ethyl ether. The ether layer washed with water

5×500 mL, dried over $MgSO_4$ and filtered, the solution is evaporated, and the residue is recrystallized from dichloromethane-methanol.

UV λ_{max} (dichloromethane) [nm] (ε [$dm^3mol^{-1}cm^{-1}$]) 236 (1.75×10^4), 270 (1.28×10^3). ^{1}HNMR ($CDCl_3$, TMS) δ 1.23 (t, 6H, J=7.2 Hz); 3.19 (d, 2H, J=21.9 Hz); 3.90 (s, 3H); 4.02 (q, 4H, J=7.2 Hz); 7.36 (d, 2H, J=8.1 Hz); 7.98 (d, 2H, J=8.1 Hz). ^{13}CNMR (75.45 MHz, $CDCl_3$, TMS) δ 16.31 (d, Jpocc=5.4 Hz, $-CH_3$); 34.00 (d, Jpc=138.5, $-OPCH_2-$); 52.06 ($-OCH_3$); 62.52 (d, Jpoc=7.6 Hz, $-CH_2OPO-$); 128.78 ($1C_{Ar}$); 129.75 ($2CH_{Ar}$); 129.84 (d, $J_{PCH2CArCAr}$=6.4 Hz, $2CH_{Ar}$); 137.17 (d, $J_{PCH2CAr}$=8.6 Hz, $1CH_{Ar}$); 166.88 ($-COOCH_3$). MS [m/z] 286.1 (M^+).

Synthesis of Quenchers--Carotenide succinimide ester

The carotenide acid 100mg dissolved in 4 mL DMF added into 42.12 mg DCC. The mixture was stirring at room temperature under argon. After 6 hrs. added into N-hydroxysuccinimide 21.5 mg. After 20 hrs. removed DMF. UV λ_{max} (dichloromethane) [nm] (ε [$dm^3mol^{-1}cm^{-1}$]) 236 (1.75×10^4), 270 (1.28×10^3). ^{1}HNMR ($CDCl_3$, TMS) δ 1.23 (t, 6H, J=7.2 Hz); 3.19 (d, 2H, J=21.9 Hz); 3.90 (s, 3H); 4.02 (q, 4H, J=7.2 Hz); 7.36 (d, 2H, J=8.1 Hz); 7.98 (d, 2H, J=8.1 Hz). ^{13}CNMR (75.45 MHz, $CDCl_3$, TMS) δ 16.31 (d, Jpocc=5.4 Hz, $-CH_3$); 34.00 (d, Jpc=138.5, $-OPCH_2-$); 52.06 ($-OCH_3$); 62.52 (d, Jpoc=7.6 Hz, $-CH_2OPO-$); 128.78 ($1C_{Ar}$); 129.75 ($2CH_{Ar}$); 129.84 (d, $J_{PCH2CArCAr}$=6.4 Hz, $2CH_{Ar}$); 137.17 (d, $J_{PCH2CAr}$=8.6 Hz, $1CH_{Ar}$); 166.88 ($-COOCH_3$). MS [m/z] 286.1 (M^+).

Synthesis of Conjugates--BChl—Molecular Beacons

After BChl-NCS, model BChl (Pyro-succ) and Car precursors were synthesized in order to construct a BChl-MBs. Pyro acid was used in place of BChl-NCS for a model study (Figure 13 shows the molecular structure of BChl-MBs).

Synthesis of Conjugates--Method for Synthesis of model BChl--MBs

By this method, successive synthesis a Pyro-30mer-Car through solid phase reaction (Figure 14) is performed. The model oligonucleotide (**5'-GCGAGTCCCGCCTGTGACATGCATTCTCGC-3'**; (SEQ ID NO:8)) includes a 20mer AS-ON sequence identical to ISIS 5132, which is a *c-raf* kinase AS-ON currently in Phase II study for various cancers, and with two 5mer arm sequences(underlined) at the each end of the sequence which are complementary each other to form stem of MB. Pyro and Car were attached to the end of each arm respectively. Figure 14 shows the molecular structure of Pyro-30mer-Car.

Synthesis of Conjugates--Pyro –CPG synthesis

Pyropheophorbide acid (22mg.40umol) was dissolved in 3mL DMF and activated with HBTU(15.2mg, 40umol)/HOBt (5.5mg,40umol) under the presence of argon for 20min. The intermediate mixture was transferred to the shake flask containing 3'-Amino-Modifier C_3 CPG (300mg, Fmoc loading >25umol/g, Glen Research) CPG, of which the Fmoc protecting group was removed with 20% piperidine/DMF in advance. After shaking the flask at room temperature for 12h, the CPG was filtered and washed with DMF (3×5mL), ACN(3×5mL) and DCM (3×5mL) successively to remove unreacted reagents. The CPG was then capped with acetic anhydride /pyridine in THF (10% solution) for 1h, following by washing with DMF, CH_3CN to afford Pyro modified CPG. MS [m/z] 966.5(M^+).

Synthesis of Conjugates--Pyro-oligonucleotide-MMT synthesis

On an automatic DNA synthesizer, the first 3'-nucleotide G is covalently attached to the Pyro-modified CPG and successive nucleotide monomers are added one by one through a cycle of four chemical reactions: detritylation, coupling, capping and oxidation. After finishing the DNA sequence synthesis, a 5'-Amino-modifier C3 was anchored to the DNA sequence at the last coupling step to afford Pyro-30mer-MMT. Figure 16 shows the absorption spectrum of Pyro modified CPG(Green line) and Pyro-30mer(red line) ,which were cleaved from solid support.

Synthesis of Conjugates--Pyro-car-Car synthesis

Activated Car-acid (22.2mg, 40umol) was incubated with HBTU(15.2mg.40umol) and HOBt (5.5mg,40umol) in DMF under the presence of argon for 20min. The intermediate mixture was transferred to a flask containing Pyro-30mer-CPG(8umol), of which the MMT protect group of the 5'-Amino-modifier was removed by 2.5% TCA in DCM. After shaking overnight at room temperature in the presence of argon, the CPG was filtered and washed with NMF (3×5mL), DCM(3×5mL) and MeOH (3×5mL). The oligonucleotide was cleaved from CPG in ammonium hydroxide at 55°C for 17hr and the solution dried followed by purification of the compound by HPLC to produce Pyro-DNA-Car. (HPLC method: Using 0.1M TEAA and CH_3CN as HPLC eluent, from10%CH_3CN to 90% CH_3CN for 45min.) Figure 17 shows the HPLC result and the absorption spectrum of Pyro-30mer-Car(blue line).

Synthesis of Conjugates--Alternative Method for Synthesis of model BChl--MBs

Using the commercial Pthalimidyl-modified CPG (TrilinkBiotechnologies.Co), oligonucleotide synthesis using an automated DNA synthesizer is performed first. Following synthesis, a 5'-Amino-modifier C3 was anchored to the DNA sequence at the last DNA synthesis step. After removing the MMT protecting group of 5'-Amino-modifier C3, Carotenide acid was conjugated to the DNA sequence with HBTU/HOBt activation. After cleaving this oligonucleotide from CPG and removing Pthalimidyl (Pth)protected group in concentrated ammonium hydroxide at 55C for 17h, the Pyro NHS was conjugated to the DNA sequence by solution reaction. Scheme6 showed this synthesis process

Synthesis of Conjugates--Pthalimidyl modified DNA sequence synthesis

Using an automatic DNA synthesizer, the first 3'-nucleotide G is covalently attached to the Pth-modified CPG and successive nucleotide monomers are added one by one through a cycle of four chemical reactions: detritylation, coupling, capping and oxidation. After finishing the DNA sequence synthesis, a 5'-Amino-modifier C3 was anchored to the DNA sequence at the last coupling step to afford Pth-30mer-MMT.

Synthesis of Conjugates--Pth-oligonucleotide-Car synthesis

Activated Car-acid (22.2mg, 40umol) was incubated with HBTU(15.2mg.40umol) and HOBt (5.5mg,40umol) in DMF under the presence of argon for 20min. This intermediate mixture was transferred to flask containing Pth-30mer-CPG(8umol), of which the MMT protect group of the 5'-Amino-modifier was removed by 2.5% TCA in DCM. After shaking overnight at room temperature in the presence of argon, the CPG was filtered and washed with DMF (3×5mL), DCM(3×5mL) and MeOH (3×5mL). The oligonucleotide was cleaved from CPG in ammonium hydroxide at 55°C for 17hr and the Pth protected group was removed in this step, and then dried after filtration followed by purifying the compound using HPLC to afford NH_2-30mer-Car. (HPLC method: Using 0.1M TEAA and CH_3CN as HPLC eluent, from30%CH_3CN to 70% =CH3CN for 45min.) Figure 18 shows the HPLC retention time and absorption spectrum of NH_2-30mer-Car.

Synthesis of Conjugates--Pyro-oligonucleotide-Car synthesis

The Pyro-NHS was reacted with the NH_2-30mer-Car in DMSO in the presence of DIPEA for 10hr at room temperature. After HPLC purification, the Pyro-DNA-Car substrate was isolated. Figure 19 shows the HPLC profile. The reaction yield is more than 40%. (See Figure 20).

Example 2—Preparation of Conjugate with Peptide Substrate

Synthesis of Conjugates--Synthesis of peptide PDT beacon

PDT beacons consisting of peptide sequences which can be cleaved by specific enzymes overexpressed in tumor cell were also designed and synthesized.

Synthesis of Conjugates--Synthesis of Model BChl-Peptide PDT Agents:

A cleavable caspase-3 substrate GDEVDGSGK (SEQ ID NO: 18; cleavage site underlined) was chosen as the peptide sequence, for which there is a well-established assay for the caspase-3 specific fluorogenic substrate. Based on the same reason as for Bchl-MBs, pyropheophorbide acid was used instead of BChl to synthesize the peptide PDT beacon. Figure 21 shows this synthesis process.

Synthesis of Conjugates--Synthesis of Caspase-3 cleavable peptide sequence

Caspase -3 substrate G*DEVD*GSGK(Mtt) (SEQ ID NO: 18) with Glycine and lysine at both ends for conjugation was synthesized by manual Fmoc SPPS (solid phase peptide synthesis) protocol using sieber amide resin and O-(Benzotriazole-1-yl)-N,N,N',N'-tetramethyluronium-hexafluorophosphate (HBTU) /1-Hydroxybenzotriazole(HOBt) as the activating reagents. Every step of peptide synthesis was monitored by EMS spectrum and HPLC chromatograph in order to get enough purified peptide sequence (purity is more than 90%,see Figure 22 HPLC result.).

Synthesis of Conjugates--Synthesis of PS conjugated peptide sequence

Activated Pyro-acid was incubated with HBTU and HOBt in NMP under the presence of argon for 20min. This intermediate mixture was transferred to a flask containing peptide-resin , of which the Fmoc protected group of the last amino acid (Glycine) was removed by 20% Piperidine in DMF. After shaking overnight at room temperature in the presence of argon, the resin was filtered and washed with NMF (3×5mL), DCM(3×5mL) and MeOH (3×5mL). The peptide was cleaved in Sieber resin in 2% TFA in DCM followed by treatment with a deprotection solution: (30:5:65TFA/Triisopropylsilane/DCM) for 1hr. The solution was concentrated and the compound precipitated in ether to produce a green cotton-like solid. The composition of this compound was confirmed by matrix –assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF) Calcd. 1378.49, found 1378.84.The purity is more than 90% from HPLC(see Figure 23).

Conjugation of Quencher to the PDT substrate

The Mtt protect group on lysine was removed during the peptide cleavage and deprotection. The Car-NHS was then reacted with the lysine residue of the opposite end of the peptide chain (Figure 21). Purification of this product was achieved by using two connected Diol and C18 Sep-pak columns and confirmed by matrix –assisted laser desorption ionization time-of-fight mass spectrometry (calculated: 1895.24, actual: 1896.03). The presence of all three structural components in PPC was further confirmed by absorption spectroscopy (Figure 24C), which shows characteristic peaks of the Pyro (419, 664nm) and Car (470, 500nm) moieties.

The activation of PPC

The PPC was then tested for caspase-3 cleavage using HPLC. As showed in Figure 25A, addition of caspase-3 clearly induced cleavage, as demonstrated by the diminishing PPC peak at 27min and the rise of Pyro-peptide (PP) peak at 13 min. The cleavage was proved to be caspase-3-specific by using a caspase-3-specific inhibitor (Ac-DEVD-CHO) that completely blocked the enzyme activity. Another observation is that the caspase-3 cleavage of PPC also led to two to three fold fluorescence enhancement, indicating that, in addition to $^1O_2$ quenching, Car is capable of quenching Pyro fluorescence to some extent. This could allow these agents to serve both as directed-PDT and tumor-specific diagnostic agents.

To test this concept, $^1O_2$ was measured directly in solutions of PPC alone, PPC incubated with caspase-3 and PPC incubated with caspase-3 plus a caspase-3 inhibitor. PP alone, without the Car moiety, was used as a positive control. $^1O_2$ generation was quantified by measuring its near-infrared (NIR) luminescence at 1270 nm. Briefly, a 10 ns pulsed 532 nm laser excites the solution and the luminescence spectrum is sampled using a set of interference filters and a high-sensitivity NIR photomultiplier tube operating in time-resolved single photon counting mode, after rejection of PS fluorescence.

As shown in Figure 25B and 25C, addition of caspase-3 to the PPC (molar ratio: 1:60; incubation time: 1 hour) resulted in a four-fold increase in $^1O_2$ signal, an effect that was completely reversed by co-incubation with the caspase-3 inhibitor (8 x PPC concentration with the same incubation time). As expected, the $^1O_2$ generation by PP was unaffected by caspase-3 or its inhibitor. The two-fold difference in luminescence between the PP and PPC+caspase-3 was related to a corresponding decrease in the $^1O_2$ lifetime, probably due to the presence of free CAR quenchers in solution after cleavage. The difference in $^1O_2$ luminescence between PPC alone and PPC+caspase-3 is likely due to both photosensitizer triplet-state and $^1O_2$ quenching by CAR. Hence, these data demonstrate that $^1O_2$ generation is effectively inhibited by the CAR quencher and that caspase-3-induced separation of the quencher and the photosensitizer molecules allows photoactivation of the latter. All of the above experiments were repeated at least in triplicate and were statistically significant ($p<0.04$).

Example 3—Delivery of Conjugate with Nucleic Acid Substrate

Determination of the hybridization efficiency in solution

It will be demonstrated that a model BChl-MB can hybridize with a ssDNA (5'-AATGCATGTCACAGGCGGGA-3'; SEQ ID NO: 19) that is complementary to the loop sequence of MB. Because detection sensitivity for $^1O_2$ is much lower than the sensitivity of fluorescence, it the hybridization efficiency will first be determined by spectrofluorimetry. Thus, the model BChl-MB or control MBs will be added to an excess (5-10 fold) of complementary target DNA. The efficiency of energy transfer between the BChl and the Car will be determined by quantifying the fluorescence intensity of the BChl at 840nm using the excitation wavelength of 825nm.

Determination of the hybridization efficiency in solution--Delivery of BChl-MBs to cells expressing target mRNA

Since the majority of mRNA is located in the cytoplasm instead of the nucleus, it is desirable to deliver MBs to the intracellular compartments (preferably cytosol). Accordingly, efficient delivery of the BChl-MBs to the target cells will be accomplished. First, transfection with cationic lipids such as Lipofectamine will be used because they are the most widely used vehicles for in vitro AS-ON delivery. These cationic lipids are positively charged molecules that bind negatively charged ONs through electrostatic interactions. Then, transport-enhancing peptides will be utilized to improve cytoplasmic delivery. Several peptides including ANT, a 16 amino acid sequence from *Drosophila* antennapedia protein, are actually transported across the cellular membrane and localize in the cytoplasm. During intracellular delivery, nuclease mediated degradation of BChl-MBs may occur. Accordingly, BChl-MBs can be constructed using thiolated AS-ONs, since these thiolated AS-ONs are less susceptible to nuclease degradation.

Determination of the hybridization efficiency in solution--Transfection of BChl-MBs with Lipofectamine

Lipofectamine 2000 (LF2000), a reagent from Invitrogen Corp. (Carlsbad, CA), is suitable for the transfection of nucleic acids into eukaryotic cells. BChl-MB-LF2000 complexes are prepared following standard protocols and are added directly to cells in culture medium. Three cell lines will be studied. For model BChl-MBs targeting c-raf-1 mRNA, the MDA-MB-231 breast cancer cell line will be used. For EGFRvIII mRNA targeting BChl-MBs, HC20 cells (EGFRvIII positive) and C012 cells (EGFRvIII negative) both derived from NIH-3T3 cells, will be provided be used. The efficiency of intracellular delivery of BChl-MBs will be determined by fluorescence confocol microscopic study.

Determination of the hybridization efficiency in solution--Transport-enhancing peptides

BChl-MBs will be synthesized with ANT peptide sequence incorporated via an S-S linkage into the 5'-end of the MB to serve as an intracellular delivery vehicle (see Scheme 2), with the assumption that its disulfide bond will be cleaved before the MB enters the nucleus, thus achieving the cytosol delivery.

Determination of the hybridization efficiency in solution--$^1O_2$ measurement in solution and in cells in vitro

Because of the strongly decreased lifetime of $^1O_2$ in cells and tissues caused by rapid quenching by biomolecules, a reliable $^1O_2$ measurement assay in cells has not been available until recently. A system based on a high-sensitivity NIR photomultiplier tube, with corresponding light activation and detection protocols for measuring $^1O_2$ luminescence in cells *in vitro* and in tissues *in vivo* has been developed (Dr. Brian Wilson; Consortium Investigator, University of Toronto). This system will be available to measure $^1O_2$ generation, not only in solution but also in tumor cells *in vitro* and subsequently tumors *in vivo*. PDT generation of $^1O_2$ will be made for the model BChl-MB upon hybridization with corresponding targets.

Determination of the hybridization efficiency in solution--Identify suitable loop using MB probes and computer programs

Hybridization to total RNA extracted from EGFRvIII mRNA expressing HC20 cells will be performed using standard procedures. Total mRNA will be extracted by following Chomczynski and Sacchi' single step extraction method. RNA (3μg) will be heated to 95°C for 5 minutes, then hybridized to MB probes. After hybridization overnight at 37°C, 0.6 ml of Tris buffer containing 1 mM $MgCl_2$ will be added and samples will be centrifuged for 20 minutes to remove particulate matter. Emission spectra will be scanned with a fluorimeter as described above. Detection of a strong fluorescence signal only when MBs were placed in solution with the ON target to which they could hybridize is expected. Thus, the desired loop sequence for proposed breast cancer-specific BChl-MB, EGFRvIII AS, will be defined. In addition, computer programs ("MFOLD" or "foldsplit") that calculate the local folding potential for a given mRNA sequence will be used to facilitate the mapping process.

Once adequate EGFRvIII mRNA targets have been identified the desired EGFRvIII AS loop containing BChl-MBs will be synthesized using the methods described above. BChl-MBs will be delivered into HC20 cells (EGFRvIII positive) and C012 cells (EGFRvIII negative) following LF2000 transfection. Once the MB is delivered to its subcellular target, the hybridization process between BChl-MB and the target mRNA occurs and enables the $^1O_2$ production. This process will be confirmed by the direct $^1O_2$ detection assay as described above.

The lifetime of BChl-MB/EGFRvIII mRNA hybrid depends on factors such as RNase-H activity. If this hybrid is cleaved by RNase-H, BChl-MB returns to its hairpin form and the $^1O_2$ is quenched. In other words, the therapeutic window for the PDT treatment no longer exists. Therefore, this study will determine the optimum PDT treatment time *in vitro*. Thus, BChl-MBs will be first incubated with HC20 cells and suitable delivery vehicles. For multiple time points during incubation, cells will be collected to measure MB uptake by spectrofluorimetry. The time point of highest fluorescence will be identified and used. Measurements at the same time points in C012 cells will be used to determine when fluorescence begins to appear in this cell line. This will be used as an indication of when degradation of the MB begins to occur.

In vitro determination of the photodynamic efficacy and selectivity of BChl-MB PDT.

These experiments test the utility of the EGFRvIII mRNA triggered BChl-MB PDT to treat breast cancer. A BChl-MB construct that exhibits selective photosensitization of its target cells when hybridization has taken place will be identified.

To determine the ability of BChl-MB to photosensitize cells expressing EGFRvIII mRNA, the clonogenic survival of BChl-MB PDT-treated HC20 cells will be compared with the survival of C012 cells subjected to the same PDT protocol. Thus cells will be exposed to the BChl-MB and delivery vehicle for the incubation time required for maximum binding of BChl-MB as described above. Following this incubation, the BChl-MB-containing media will be removed, and cells will be rinsed in HBSS, trypsinized to detach them from the plate and illuminated in suspension. PDT will be carried out with a diode laser emitting at 830 ± 10 nm. The light dose rate will be measured using an isotropic detector based light dosimetry system. Immediately after illumination, cells will be plated at concentrations from $10^2 - 10^6$ cells/ 100 mm dish in their standard growth media and incubated in 5% CO_2 until colony formation. For each cell line, survival curves will be created as a function of "equivalent drug dose" and survival parameters will be determined and statistically compared using the JMP software (SAS Institute, Inc., NC).

The expected results are an effective PDT response to BChl-MB in the EGFRvIII-expressing cell line, with no response in the non-expressing cells. If (non-EGFRvIII dependent) cell kill is found in C012 cells, the enhancement of kill in the HC20 line will be quantified as the ratio of the drug or light dose required to produce a 90% reduction in the surviving fraction of C012 cells to HC20 cells.

The specificity of BChl-MB PDT is provided by the precise targeting of BChl-MB to the EGFRvIII mRNA and the necessity for drug binding for $^1O_2$ production to take place. Survival curves will be performed as described above, with the exception that after detachment from the plate cells, the cells will be resuspended in the photosensitizer-containing media for illumination. In parallel studies, PDT will be carried out with an equivalent dose of free BChl, also with illumination taking place in the presence of the photosensitizer. Survival curves will be determined to compare the change in survival when illumination is performed in the presence *versus* absence of BChl-MB and when illumination is performed in the presence *versus* absence of free BChl. Cells illuminated in the absence of drug will be exposed to drug during incubation but the drug containing media will be removed before light exposure. Results will be quantified as the ratio of the dose (drug or light) necessary to create a 90% reduction in clonogenicity for illumination in the absence of drug to the dose required for illumination in the presence of drug.

The expected results are a decrease in clonogenicity when PDT is performed in the presence of free BChl compared to illumination in the absence of free BChl.

Example 4—Preparation of a Conjugate with PSA-Cleavable Substrate

Synthesis of a PSA-cleavable photodynamic therapy (PDT) agent with a HSSKLQ-containing peptide sequence, a pyropheophorbide (Pyro) photosensitizer (PS), and a singlet oxygen ($^1O_2$) quencher/scavenger (Q).

The most important requirement for a desired PSA-specific substrate is the efficient cleavage by PSA. It has been shown that doxorubicin can be coupled directly to the Ac-HSSKLQ (SEQ ID NO:20) peptide to form the prodrug Ac-HSSKLQ-Dox. Using HPLC detection, it was determined that PSA was unable to hydrolyze the amide bond between the doxorubicin amine and the C-terminal glutamine of the peptide. On the other hand, incubation of the Mu-HSSKLQ-Leu-Dox prodrug (Mu = morpholinocarbonyl) with enzymatically active PSA resulted in production of Leu-Dox demonstrating that the steric hindrance introduced by the bulky doxorubicin is a major factor to prevent the PSA-induced peptide cleavage. In the latter construct, Mu was chosen as the NH_2 terminal blocking group because of its stability and to enhance substrate solubility. Since both Pyro photosensitizer and CAR quencher are both large molecules (molecular weight ~600) with rigid structures comparing with Mu (molecular weight 115), a Pyro-GGHSSKLQGSGK-CAR PDT (SEQ ID NO: 21) beacon containing a 12 amino acid sequence is proposed. The rationale of this design is as follows: 1) CAR is acid-labile, thus it should be coupled to the peptide in the final conjugation in solution; 2) A C-terminal lysine residue is required for CAR conjugation; 3) The increasing number of glycine residues on both side of the PSA-specific sequence is to compensate the steric hindrance of Pyro and CAR moiety to allow a better folding scaffold for maximizing the interaction between the PS and Q; 4) The inclusion of serine residue is to enhance the substrate solubility to compensate the increase in hydrophobicity introduced by Pyro and CAR moieties.

Peptide PDT beacon synthesis

The proposed synthetic pathway for Pyro-GGHSSKLQGSGK-CAR (PPC) (SEQ ID NO: 21) PDT beacon is depicted in Figure 27. Briefly, GGHSSKLQGSGK peptide (SEQ ID NO: 21) will be first synthesized using a manual solid phase peptide synthesis (SPPS) protocol. Next, Pyro will be coupled to the N-terminal glycine on the solid-support. The Pyro-peptide conjugate is then cleaved from the support and deprotected. The final CAR conjugation is carried out in solution, since it is acid-labile. Another key challenge of this method is how to distinguish two lysine residues allowing CAR moiety linked to the C-terminal lysine. Therefore, an ivDde group will be used to protect the amino function of the lysine residue in the middle of the sequence, which is cleavable under a very mild condition (2% hydrazine, 5-10 minutes).

Confirmation of Cleavage of Peptide PDT by PSA

Kinetic analysis of PSA hydrolysis will be assayed by HPLC. In brief, various concentration of the beacon will be incubated in PSA buffer with enzymatically active PSA derived from human seminal plasma (CHEMICON, Canada) at room temperature. A control in PSA buffer alone will also be carried out. At discrete time points (1, 2, 4, 8 and 12 hours), aliquots of the reaction mixture are removed and analyzed by a reverse-phase HPLC (Waters, MA) equipped with a photodiode array detector and fluorescence detector. A standard curve produced by using purified free Pyro-GGHSSKLQ (SEQ ID NO: 22) peptide (PP) will be used to convert peak area to free PP concentration. Peak areas of free PP at each time point will then be converted to concentration, and the concentration data will be analyzed by Lineweaver-Burke plots (1/V versus 1/S, where V = reaction velocity and S = substrate concentration). K_m, V_{max} and K_{cat} will be calculated from these plots, and the ratio of K_{cat} to K_m will be used to compare hydrolysis of the PDT beacon with hydrolysis of the reported fluorogenic PSA substrate Mu-HSSKLQ-AMC (SEQ ID NO:2), where AMC is 7-amino-4-methyl coumarin (Voigt Global, Missouri).

Determination of the PSA cleavage of PDT beacon in cell culture.

To test PPF accumulation in cells, confocal microscopy was measured in human hepatoblastoma G2 (HepG2) cells. As shown in Figure 28, cells alone have no fluorescence background, whereas cells incubated with 200 μM PPF for 30 min clearly show fluorescence for both Pyro (excited at 633 nm) and FITC (excited at 488 nm). The fluorescence signals in cells grow stronger with longer incubation time (24 h). These images suggest that this kind of beacon construct can enter the cell directly without any additional delivery vehicle. Conditioned medium from LNCaP cells containing the PDT beacon will be applied to a C18 reverse-phase Bond-Elut column (Varian, CA) and washed with buffer for six column volumes consisting of 0.1M phosphoric acid and 5% acetonitrile in PBS. Samples will be eluted from the column using a solution of 70% acetonitrile/0.1% TFA (v/v). The solvents will then be evaporated to dryness, and the samples will be redissolved in 0.1% TFA (v/v) and applied to reverse-phase HPLC as described above.

Bioluminescence imaging of the efficacy of PDT agent in PSA-producing versus non-producing cells

To determine the ability of Pyro-peptide-CAR (PPC) PDT beacon to photosensitize cells producing PSA, the clonogenic survival of PPC PDT-treated isolated LNCaP cells (PSA positive) will be compared with the survival of PC3 prostate cancer cells (PSA negative) subjected to the same PDT protocol. Meanwhile, Pyro-peptide (PP) will be served as the positive control. Both prostate cancer cell lines can been stably transduced, and contain endogenous wt p53 protein with a Firefly luciferase gene together with a Renilla luciferase gene. Thus, Xenogen IVIS bioluminescence imager will be used to monitor the p53 activity together with the physiological state of the cells, which serve as a reliable indicator of PDT efficacy of the proposed PDT beacon. In brief, following the incubation, the PPC-containing media will be removed, and cells will be rinsed in HBSS, trypsinized to detach them from the plate and illuminated in suspension. PDT will be carried out with a laser emitting at 670 nm. The light dose rate will be measured using an isotropic detector based light dosimetry system. Immediately after illumination cells will be plated at concentrations from 10^2 – 10^6 cells / 100 mm dish in their standard growth media and incubated in 5% CO_2 until colony formation. For PSA-producing LNCaP cells, the expected results are the increase of p53 transcription activity determined by Firefly luciferase bioluminescence imaging and the cell death observed by Renilla luciferase bioluminescence. For PSA non-producing PC3 cells, an opposite observation is expected.

Bioluminescence imaging of the efficacy of PDT beacon in PSA-producing versus non-producing tumor xenografts

Since bioluminescence imaging is at its best for real-time, non-invasive live small animal imaging, changes in tumor volume will be monitored by Renilla luciferase luminescence as well as changes in p53-dependent transcriptional activity by Firefly luciferase luminescence in mice following *in vivo* PDT treatment. The expected results are the tumor shrinkage and the increased p53 activity in PSA-producing LNCaP xenograft model observed through Renilla and Firefly luciferase, respectively, whereas none of these should change significantly in the PSA non-producing PC3 xenograft model.

Example 5—Preparation of Conjugates with Death Sensor

Enzyme-activated PDT Agent with a Built-In Cell Death Sensor (Bifunctional Smart PDT Agents)

The concept underlying this invention is depicted in Figure 3. In general, these new "smart" PDT agents should have following characteristics:

1) they will contain a PDT agent (P) that can both can generate singlet oxygen and emit fluorescence upon light activation

2) they will also contain two substrate sequences, the second substrate will be a cell death marker, for example, a well known apoptosis marker. Accordingly, a second substrate such as a caspase substrate, (for: caspase-1, caspase-3, caspase-9, *etc.*) will be used, and the first substrate will be a tumor-specific substrate including but not limited to, peptides, nucleic acids and synthetic polymers, which can be specifically cleaved by certain enzymes;

3) these agents will place P between the two sequences described above; a singlet oxygen quencher (Q_s) will be attached to the terminal end of the tumor-specific substrate and a fluorescence quencher (Q_f) will be attached to the terminal of the caspase substrate; and

4) both P and Q_s and P and Q_f are held in proximity by the appropriate length of the substrate sequence. Therefore, when both sequences are intact, Q_s quenches the singlet oxygen produced by P and Q_s quenches the fluorescence of P. Thus, PDT treatment will not harm normal cells, and no fluorescence signal will be observed.

However, when these agents enter cancer cells, the tumor-specific substrate will undergo a change of conformation such that Q_s will be removed from the immediate vicinity of P. In certain embodiments the change of conformation is cleavage of the tumor-specific substrate. Upon photoirradiation (i.e., PDT treatment), P will generate singlet oxygen, which will kill the cancer cells. Moreover, if the PDT treatment is effective, apoptosis is expected to occur within the cancer cells; this process will produce caspases. These enzymes will cleave the sequence between P and Q_f, and once Q_f is removed from the immediate vicinity of P, the quenched fluorescence will be restored. If the PDT treatment is ineffective, there would be no apoptosis. Thus, there would be no caspase activity and the caspase substrate would remain intact, and no fluorescence would be observed. Through this design, not only will the PDT agent be activated exclusively in cancer cells leaving normal cells unharmed, but the effectiveness of such smart PDT agents would also be monitored noninvasively *in vivo* in real time, providing an immediate monitoring of therapeutic outcome.

Tumor-specific enzyme substrate

Prostate-specific antigen (PSA) is a serine protease secreted by both normal prostate glandular cells and prostate cancer cells. It is found in high concentration in the seminal plasma, where the major proteolytic substrates for PSA are the gel-forming proteins in freshly ejaculated semen, SgI and SgII. On the basis of the PSA cleavage map for SgI and II, a peptide with the amino sequence His-Ser-Ser-Lys-Leu-Gln (HSSKLQ; SEQ ID NO:2) was identified by Isaacs *et al.* that had a high degree of specificity for PSA. This substrate is used to demonstrate that prostate cancer cells secrete enzymatically active PSA into the extracellular fluid and that PSA becomes inactivated by serum protease inhibitors on entering the blood. On the basis of this information, others developed a PSA-activated doxorubicin prodrug (HSSKLQ-Leu-Dox, L-377202, Merck; SEQ ID NO:2) that is inactive when given systemically but becomes activated when processed proteolytically within prostate cancer metastases by PSA. Therefore, this PSA substrate, HSSKLQ peptide (SEQ ID NO:2), is chosen to build a "smart" PDT agents targeting prostate cancer.

Caspase substrate

Recognition of the central role of caspases in the programmed cell death process (apoptosis) has led to the development of assays that can measure these important enzymes *in situ*. Caspase activation represents one of the earliest known markers for the onset of apoptosis. In most instances, caspase activation precedes cell permeability alterations and DNA damage, whereas cytoskeletal collapse and phosphatidylserine (PS) flipping are often more concurrent. Loss of mitochondrial membrane generally occurs prior to caspase activation. Several fluorogenic assays have been developed for *in situ* analysis of caspase activation in intact cells. These assays are useful for detecting localized caspase activation in early apoptotic cells. Among these assays, caspase-3 fluorogenic substrates with the common DEVD cleavage site are the most widely utilized markers for identifying the early critical onset of cancer cell apoptosis. Therefore, this enzyme substrate, DEVD peptide, is chosen to integrate a cell death sensor into a "smart" PDT agent to monitor the effectiveness of the agents in real time.

The structure of the first construct for this concept is depicted in Figure 25. It contains a pyropheophorbide (Pyro) as P, a carotenoid (Car) as Q_s, a black hole quencher (BHQ) as Q_f, with a PSA substrate between P and Q_s and a caspase-3 substrate between P and Q_f. This conjugate is named BHQ-GDEVDSGK(Pyro)HSSKLQK-Car (GDEVDSGKHSSKLQK is SEQ ID NO:23).

Example 6—Preparation of Conjugate with Phospholipid as Substrate

A PLA_2-specific phospholipid probe is synthesized incorporating both the quencher (e.g., carotenoid (Car) and the PDT agent (e.g., Pyro) into the *sn*-1 and *sn*-2 portion of the phospholipid, respectively. This design makes the release of the fluorescent moiety independent of cleavage by PLC and PLD. Furthermore, in order to have the PLA_2 specificity, the quencher is introduced into the *sn*-1 position via an ether linkage, since the ether linkage is resistant to PLA_1 and it is well known that ether-linked phospholipids also serve as substrates in phospholipase C- or D- catalyzed reactions. Thus, O-alkylation of *sn*-glycero-3-phosphocholine with N-Boc-ethylenebromide in the presence of a cesium catalyst gives a mixture of mono-*sn*-1, mono-*sn*-2 and bis-conjugate, which is separated by HPLC. The *sn*-2 resulting intermediate will be coupled to the Pyro in the presence of DCC and DMAP. After treating with trifluoroacetic acid (TFA) to remove the Boc protection group, the amino group at the *sn*-1 position is conjugated to the quencher. The resulting phospholipid, thus, is PLA_2-specific.

It will be clear that the invention may be practiced otherwise than as particularly described in the foregoing description and examples. Numerous modifications and variations of the present invention are possible in light of the above teachings and, therefore, are within the scope of the appended claims.

The entire disclosure of all publications (including patents, patent applications, journal articles, laboratory manuals, books, or other documents) cited herein are hereby incorporated by reference.

WHAT IS CLAIMED IS:

1. A conjugate comprising a first substrate, a photosensitizer, and a first quencher, said photosensitizer and said first quencher attached to said first substrate, wherein said first substrate allows said photosensitizer and said first quencher to come sufficiently close to each other to facilitate quenching of an activated form of said photosensitizer.

2. A conjugate comprising:
a first substrate;
a second substrate, said first substrate covalently linked to said second substrate;
a first quencher, said first quencher attached to said first substrate;
a second quencher, said second quencher attached to said second substrate said second quencher comprising a fluorescence quencher; and
a photosensitizer, said photosensitizer attached to said covalently linked first and second substrates, said photosensitizer comprising a fluorophore;
wherein said first substrate allows said photosensitizer and said first quencher to come sufficiently close to each other to facilitate quenching of an activated form of said photosensitizer, and wherein said second substrate allows said photosensitizer and said second quencher to come sufficiently close to each other to facilitate quenching of fluorescence from said fluorophore of said photosensitizer.

3. The conjugate of claim 1 or 2, wherein said first substrate is cleavable by an enzyme.

4. The conjugate of claim 1 or 2, wherein said photosensitizer and said first quencher are attached at opposite ends of said first substrate.

5. The conjugate of claim 4, wherein said first substrate comprises a compound selected from the group consisting of a polypeptide, a nucleic acid

molecule, a synthetic polymer, a phospholipid, a galactose containing compound, or a combination thereof.

6. The conjugate of claim 3, wherein said first substrate is a polypeptide.

7. The conjugate of claim 3, wherein said enzyme is a protease.

8. The conjugate of claim 7, wherein said protease is an aspartic proteinase.

9. The conjugate of claim 8, wherein said aspartic proteinase is selected from the group consisting of rennin, chymosin, and pepsin.

10. The conjugate of claim 9, wherein said pepsin is HIV-1 retropepsin.

11. The conjugate of claim 7, wherein said protease is selected from the group consisting of viral proteases and retroviral proteases.

12. The conjugate of claim 7, wherein said protease is selected from the group consisting of Human Immunodeficiency Virus protease, Cytomegalovirus protease, Ebstein-Barr virus protease, Hepatitis B virus protease, Hepatitis C virus protease, Herpes Simplex virus protease, Cathepsin B, Cathepsin D, a Matrix metalloproteinase, Cathepsin K, Prostate-specific antigen, Thrombin, Caspase-3, and Interleukin 1β converting enzyme.

13. The conjugate of claim 12, wherein said protease is human immunodeficiency virus type I protease.

14. The conjugate of claim 6, wherein said first substrate comprises a polypeptide and if said photosensitizer is attached to the N-terminal amino acid of said polypeptide, then said first quencher is attached to the C-terminal amino acid of said polypeptide; and if said photosensitizer is attached to the C-terminal amino acid of said polypeptide, then said first quencher is attached to the N-terminal amino acid of said polypeptide.

15. The conjugate of claim 6, wherein said first substrate comprises a polypeptide and said polypeptide comprises X-Tyr-Pro-Y, X-Lys-Lys-Y, X-Arg-Arg-Y, X-Gly-Ile-Y, X-Gly-Leu-Y, X-Ala-Ser-Y, X-Asp-Gly-Y, X-Phe-Phe-Y, X-Asp-Glu-Val-Asp-(SEQ ID NO: 1)Y, X-Gly-Pro-Arg-Y, X-Arg-Gly-Y, or X-His-Ser-Ser-Lys-Leu-Gln(SEQ ID NO:2)-Y, wherein X and Y are each independently a polypeptide comprising from one to about 15 amino acids.

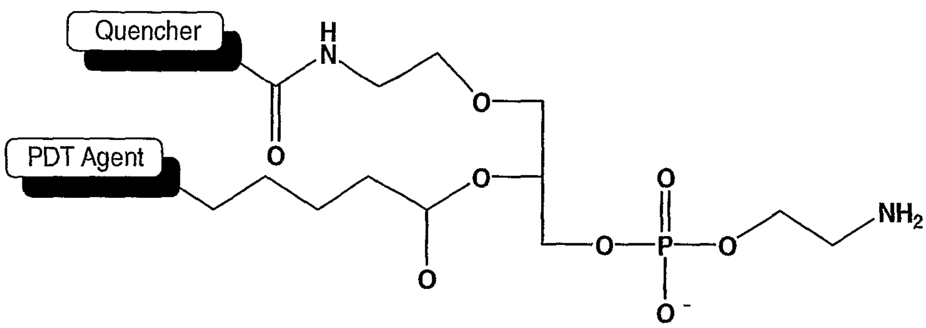
16. The conjugate of claim 15, wherein if said photosensitizer is attached to said X, then said first quencher is attached to said Y; and if said first quencher is attached to said X, then said photosensitizer is attached to said Y.

17. The conjugate of claim 1 or 2, wherein said first substrate is a phospholipid.

18. The conjugate of claim 17, wherein said phospholipid is cleavable by an enzyme.

19. The conjugate of claim 18, wherein said enzyme is Type II PLA2 (sPLA2).

20. The conjugate of claim 19, wherein said conjugate is a molecule with the following structure:



21. A conjugate comprising a first substrate, wherein said first substrate is a nucleic acid, at least one photosensitizer, and at least one first quencher, said

photosensitizer and said first quencher attached to said substrate, wherein said substrate allows said photosensitizer and said first quencher to come sufficiently close to each other to facilitate quenching of an activated form of said photosensitizer.

22. The conjugate of claim 1, 2, or 21, wherein said photosensitizer is a free base or metal complex of a compound selected from the group consisting of a pyropheophorbide, a purpurin, a porphyrin, a chlorin, a bacteriochlorin, a phthalocyanine, a naphthalocyanine, a hypericin, a porphyrin isomer, an expanded porphyrin, a cationic dye, a psoralen, and a merocyanine 540.

23. The conjugate of claim 23, wherein said expanded porphyrin is texaphyrin.

24. The conjugate of claim 22, wherein said cationic dye is rhodamine.

25. The conjugate of claim 1, 2, or 21, wherein said quencher is a carotenoid, a metal complex dye, a cyanine dye, a stilbene quinone dye, an azomethine dye, an amine, a phenol, a sulfide, a bilirubin, a biliverdin, a nitroso compound, a nitrone compound and a N-oxy compound.

26. The conjugate of claim 25, wherein said metal complex dye is nickel dithiolene.

27. The conjugate of claim 25, wherein said amine is 1,4-diazabicyclo(2,2,2)octane (DABCO).

28. The conjugate of claim 2, wherein said second substrate is cleavable.

29. The conjugate of claim 28, wherein said second substrate is cleavable by a caspase.

30. The conjugate of claim 29, wherein said caspase is selected from the group consisting of caspase-1, caspase-2, caspase-3, caspase-4, caspase-5, caspase-6, caspase-7, caspase-8, caspase-9, and caspase-10.

31. The conjugate of claim 28, wherein said first substrate and said second substrate are polypeptides, and the C- terminal amino acid of said first substrate and the N- terminal amino acid of said second substrate are covalently linked by a peptide bond.

32. The conjugate of claim 28, wherein said first substrate and said second substrate are polypeptides, and the C-terminal amino acid of said second substrate and the N- terminal amino acid of said first substrate are covalently linked by a peptide bond.

33. The conjugate of claim 31, wherein said photosensitizer is attached to either said C-terminus of said first substrate or said N-terminus of said second substrate, and said first quencher is attached to the N-terminal amino acid of said first substrate; and said second quencher is attached to the C-terminal amino acid of said second substrate.

34. The conjugate of claim 32, wherein said photosensitizer is attached to said C-terminal amino acid of said second substrate or said N-terminal amino acid of said first substrate, and said first quencher is attached to the C-terminal amino acid of said first substrate; and said second quencher is attached to the N-terminal amino acid of said second substrate

35. The conjugate of claim 28, wherein said second substrate comprises a sequence selected from the group consisting of Asp-Glu-Val-Ile(SEQ ID NO: 9), Asp-Glu-Thr-Asp(SEQ ID NO: 10), Leu-Glu-His-Asp(SEQ ID NO: 11), Asp-Glu-His-Asp(SEQ ID NO: 12), Trp-Glu-His-Asp(SEQ ID NO: 13), Leu-Glu-Thr-Asp(SEQ ID NO: 14), Asp-Glu-Val-Asp(SEQ ID NO: 15), Val-Glu-His-Asp(SEQ ID NO: 16), and Ile-Glu-Ala-Asp(SEQ ID NO: 17).

36. The conjugate of claim 35, wherein said second substrate comprises a sequence selected from the group consisting of X-Asp-Glu-Val-Ile(SEQ ID NO: 9)-Y, X-Asp-Glu-Thr-Asp(SEQ ID NO: 10)-Y, X-Leu-Glu-His-Asp(SEQ ID NO: 11)-Y, X-Asp-Glu-His-Asp(SEQ ID NO: 12)-Y, X-Trp-Glu-His-Asp(SEQ ID NO: 13)-Y, X-Leu-Glu-Thr-Asp(SEQ ID NO: 14)-Y, X-Asp-Glu-Val-Asp(SEQ ID NO: 15)-Y, X-Val-Glu-His-Asp(SEQ ID NO: 16)-Y, and X-Ile-Glu-Ala-Asp(SEQ ID NO: 17)-Y, wherein X and Y are each independently a polypeptide comprising from one to about 15 amino acids and the N-terminal amino acid of X is covalently linked to said first substrate.

37. The conjugate of claim 35, wherein said second substrate comprises a sequence selected from the group consisting of X-Asp-Glu-Val-Ile(SEQ ID NO: 9)-Y, X-Asp-Glu-Thr-Asp(SEQ ID NO: 10)-Y, X-Leu-Glu-His-Asp(SEQ ID NO: 11)-Y, X-Asp-Glu-His-Asp(SEQ ID NO: 12)-Y, X-Trp-Glu-His-Asp(SEQ ID NO: 13)-Y, X-Leu-Glu-Thr-Asp(SEQ ID NO: 14)-Y, X-Asp-Glu-Val-Asp(SEQ ID NO: 15)-Y, X-Val-Glu-His-Asp(SEQ ID NO: 16)-Y, and X-Ile-Glu-Ala-Asp(SEQ ID NO: 17)-Y, wherein X and Y are each independently a polypeptide comprising from one to about 15 amino acids and the C-terminal amino acid of Y is covalently linked to said first substrate.

38. The conjugate of claim 2, wherein said first substrate is a nucleic acid.

39. The conjugate of claim 21 or 38, wherein said nucleic acid comprises a single stranded nucleic acid.

40. The conjugate of claim 39 wherein said nucleic acid comprises a backbone selected from the group consisting of a phosphodiester, a methyl phosphonate, a phosphorothioate, a borane phosphonate, a 3'-O-phosphopropylamino, a N3'-phosphoramidate, a 2'-O-alkyl-RNA, a morpholinophosphorodiamidate, and a peptide nucleic acid.

41. The conjugate of claim 39, wherein said nucleic acid comprises a first portion, a second portion, and a third portion, wherein said first portion and said third portion are at least about 70% complementary to each other.

42. The conjugate of claim 39, wherein said first portion and said third portion are capable of base pairing with each other resulting in a stem-loop structure wherein said first portion and said third portion form the stem and said second portion forms a non-base-paired loop region.

43. The conjugate of claim 41 wherein if said quencher is attached to said first portion then said photosensitizer is attached to said third portion and if said photosensitizer is attached to said first portion then said quencher is attached to said third portion.

44. The nucleic acid of claim 41 wherein said second portion is at least about 70% identical to a nucleic acid sequence complementary to a nucleic acid sequence selected from the group consisting of c-Raf-1 mRNA, BRAF1 mRNA, DD3 mRNA, K-ras mRNA, CCND1 mRNA, and EGFRvIII mRNA.

45. The nucleic acid of claim 41, wherein said first portion is about 3 to about 7 nucleotides in length and said third portion is about 3 to about 7 nucleotides in length.

46. The nucleic acid of claim 41, wherein said second portion is about 10 to about 30 nucleotides in length

47. The nucleic acid of claim 41 wherein said first portion comprises a nucleic acid sequence consisting of SEQ ID NO: 3.

48. The nucleic acid of claim 41 wherein said second portion comprises a nucleic acid sequence selected from the group consisting of SEQ ID NOS: 5 and 6.

49. The nucleic acid of claim 41 wherein said nucleic acid comprises a nucleic acid sequence selected from the group consisting of SEQ ID NOS: 7 and 8.

50. A method of decontaminating blood comprising:
contacting blood with the conjugate of claim 1, 2, or 21 to form a blood and conjugate mixture; and
exposing said blood and conjugate mixture to an effective amount of artificial irradiation.

51. A method of inhibiting the growth of a cancer cell comprising:
(a) contacting said cancer cell with the conjugate of claim 1, 2, or 21; and
(b) exposing said cancer cell to an effective amount of artificial irradiation.

52. The method of claim 51, wherein said cancer cell is selected from the group consisting of prostate, breast, ovarian, lung, pancreatic, bladder, testicular, retinoblastoma, Wilm's tumor, adrenocarcinoma and or melonoma cells.

53. The method of claim 51, wherein said artificial irradiation is selected from the group consisting of artificial ultraviolet, infrared (IR), gamma-irradiation, x-ray and visible light.

54. The method of claim 51, wherein said artificial irradiation is applied at a wavelength ranging from about 20 nm less than the maximum absorption of the photosensitizer to about 20 nm greater than the maximum absorption of the photosensitizer.

55. The method of claim 51, wherein said artificial irradiation is applied at a rate of about 10 to about 150 mW/cm^2.

56. The method of claim 51, wherein said artificial irradiation is applied at a rate of about 35 to about 100 mW/cm^2.

57. The method of claim 51, wherein said artificial irradiation is applied at a rate of about 75 mW/cm^2.

58. A method of decontaminating blood in a subject comprising: administering to said subject the conjugate of claim 1, 2, or 21; and exposing said subject to an effective amount of artificial irradiation.

59. The method of claim 58, wherein said subject is a mammal.

60. The method of claim 58, wherein said mammal is a human subject.

61. The method of claim 58, wherein said artificial irradiation is selected from the group consisting of artificial ultraviolet, infrared (IR), gamma-irradiation, x-ray and visible light.

62. The method of claim 58, wherein said artificial irradiation is applied at a wavelength ranging from about 20 nm less than the maximum absorption of the photosensitizer to about 20 nm greater than the maximum absorption of the photosensitizer.

63. The method of claim 58, wherein said artificial irradiation is applied at a rate of about 10 to about 150 mW/cm^2.

64. The method of claim 58, wherein said artificial irradiation is applied at a rate of about 35 to about 100 mW/cm^2.

65. The method of claim 58, wherein said artificial irradiation is applied at a rate of about 75 mW/cm^2.

66. The method of claim 58, wherein the amount of conjugate administered is about 0.3 to about 3.0 mg/kg body weight of the subject.

67. The method of claim 66, wherein the amount of conjugate administered is about 1 mg/kg body weight.

68. A pharmaceutical composition comprising the conjugate of claim 1, 2, or 21 and a pharmaceutically acceptable carrier.

69. A method for the treatment of cancer in a subject comprising administering a therapeutically effective amount of the pharmaceutical composition of claim 67 to a subject in need thereof.

69. A method of treating a disease state comprising
(a) administering to a target tissue of a patient a conjugate comprising a first substrate, at least one photosensitizer, and at least one first quencher,
said photosensitizer and said first quencher attached to said first substrate,
wherein said first substrate allows said photosensitizer and said first quencher to come sufficiently close to each other to facilitate quenching of an activated form of said photosensitizer, and wherein said first substrate undergoes a change of conformation in said target tissue which stops said quenching
(b) irradiating said photosensitizer thereby killing said target tissue.

70. The method of claim 69, wherein said change of conformation is cleavage of said first substrate.

71. The method of claim 69 wherein said conjugate is a conjugate according to any one of claims 1 to 49.

72. A method of treating a disease state comprising
(a) administering to a target tissue of a patient a conjugate comprising:
a first substrate;
a second substrate, said first substrate covalently linked to said second substrate;
a first quencher, said first quencher attached to said first substrate;

a second quencher, said second quencher attached to said second substrate said second quencher comprising a fluorescence quencher; and
a photosensitizer, said photosensitizer attached to said covalently linked first and second substrates, said photosensitizer comprising a fluorophore;
wherein said first substrate allows said photosensitizer and said first quencher to come sufficiently close to each other to facilitate quenching of an activated form of said photosensitizer, and
wherein said second substrate allows said photosensitizer and said second quencher to come sufficiently close to each other to facilitate quenching of fluorescence from said fluorophore of said photosensitizer wherein said first substrate undergoes a change of conformation in said target tissue thereby, and
(b) irradiating said photosensitizer killing said target tissue,
wherein said second substrate is cleaved by a protease which removes said second quencher from said conjugate and allows fluorescence from said fluorophore of said photosensitizer to be detected.

73. The method of claim 72, wherein said change of conformation is cleavage of said first substrate.

74. The method of claim 69 wherein said conjugate is a conjugate according to any one of claims 2 to 49.

75. A method of treating a disease state comprising
(a) administering to a target tissue of a patient a conjugate comprising:
a first substrate comprising a nucleic acid wherein said nucleic acid comprises a first portion, a second portion, and a third portion, said first portion and said third portion capable of base-pairing resulting in a stem-loop structure wherein said first portion and said third portion form the stem and said second portion forms a non-base-paired loop region,
at least one photosensitizer, and
at least one first quencher, said photosensitizer and said first quencher attached to said first substrate, said first substrate capable of bringing said photosensitizer and said first quencher sufficiently close to each other to

facilitate quenching of an activated form of said photosensitizer wherein said first substrate undergoes a change of conformation in said target tissue such that formation of said stem-loop structure is inhibited, and

(b) irradiating said photosensitizer thereby killing said target tissue.

76. The method of claim 75, wherein said change of conformation of said first substrate is facilitated by the annealing of said second portion to a nucleic acid present in said target tissue.

77. The method of claim 75 wherein said conjugate is a conjugate according to any one of claims 21 and 38 to 49.

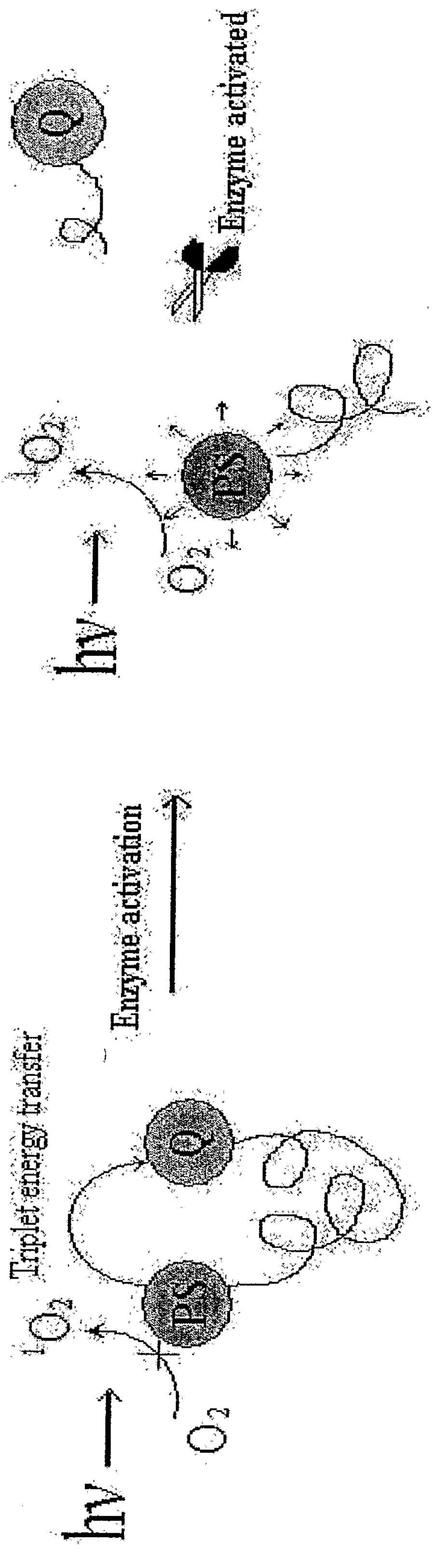

hv
O_2
$^1O_2$
PS
Q
Triplet energy transfer
Enzyme activation
hv
O_2
$^1O_2$
PS
Q
Enzyme activated

Figure 1

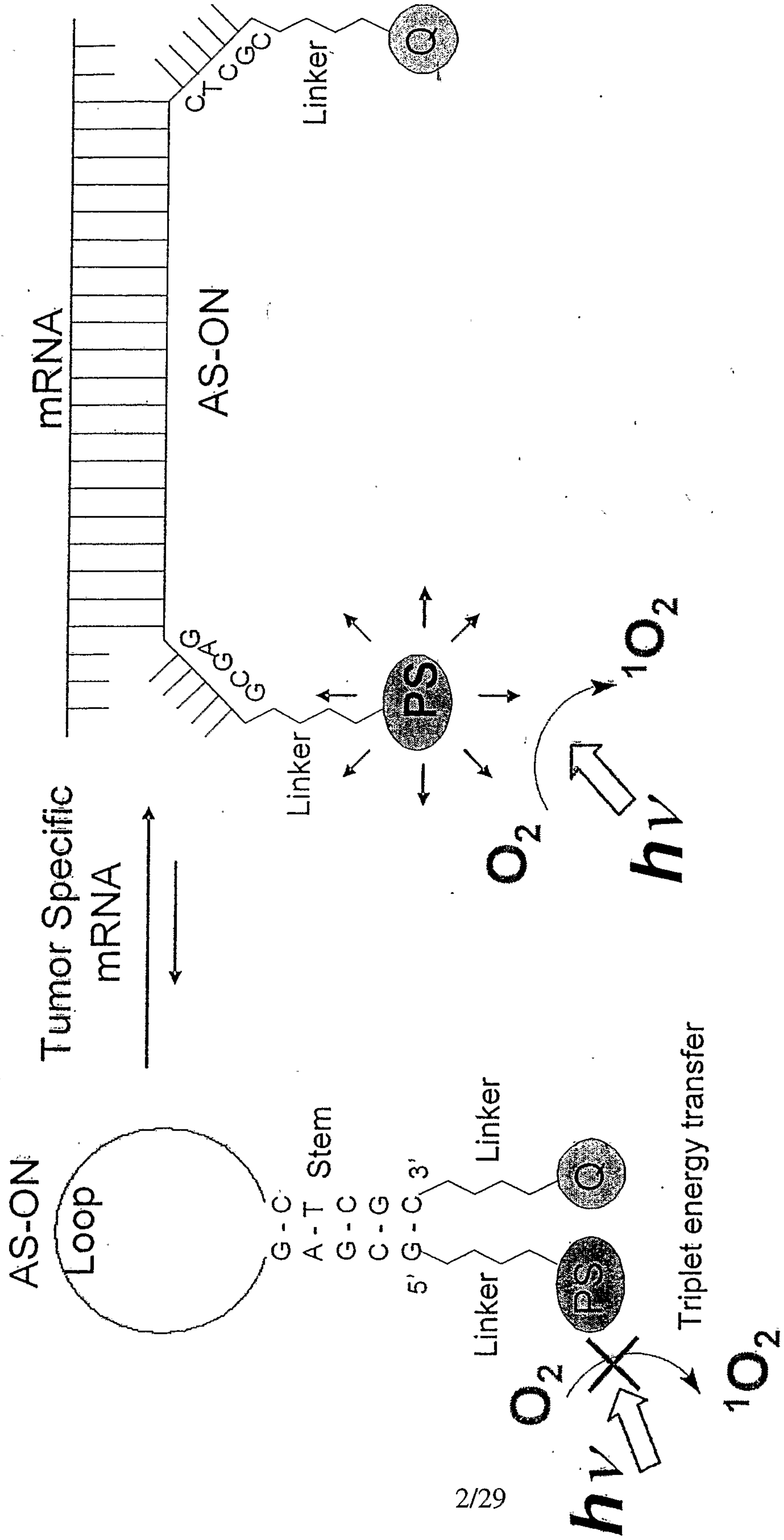

AS-ON
Loop
Stem
G - C
A - T
G - C
C - G
5' G - C 3'
Linker
Linker
PS
Q
Triplet energy transfer
O_2
$^1O_2$
$h\nu$
Tumor Specific
mRNA
mRNA
AS-ON
CTCGC
GAGCG
Linker
Linker
Q
PS
O_2
$^1O_2$
$h\nu$

Figure 2

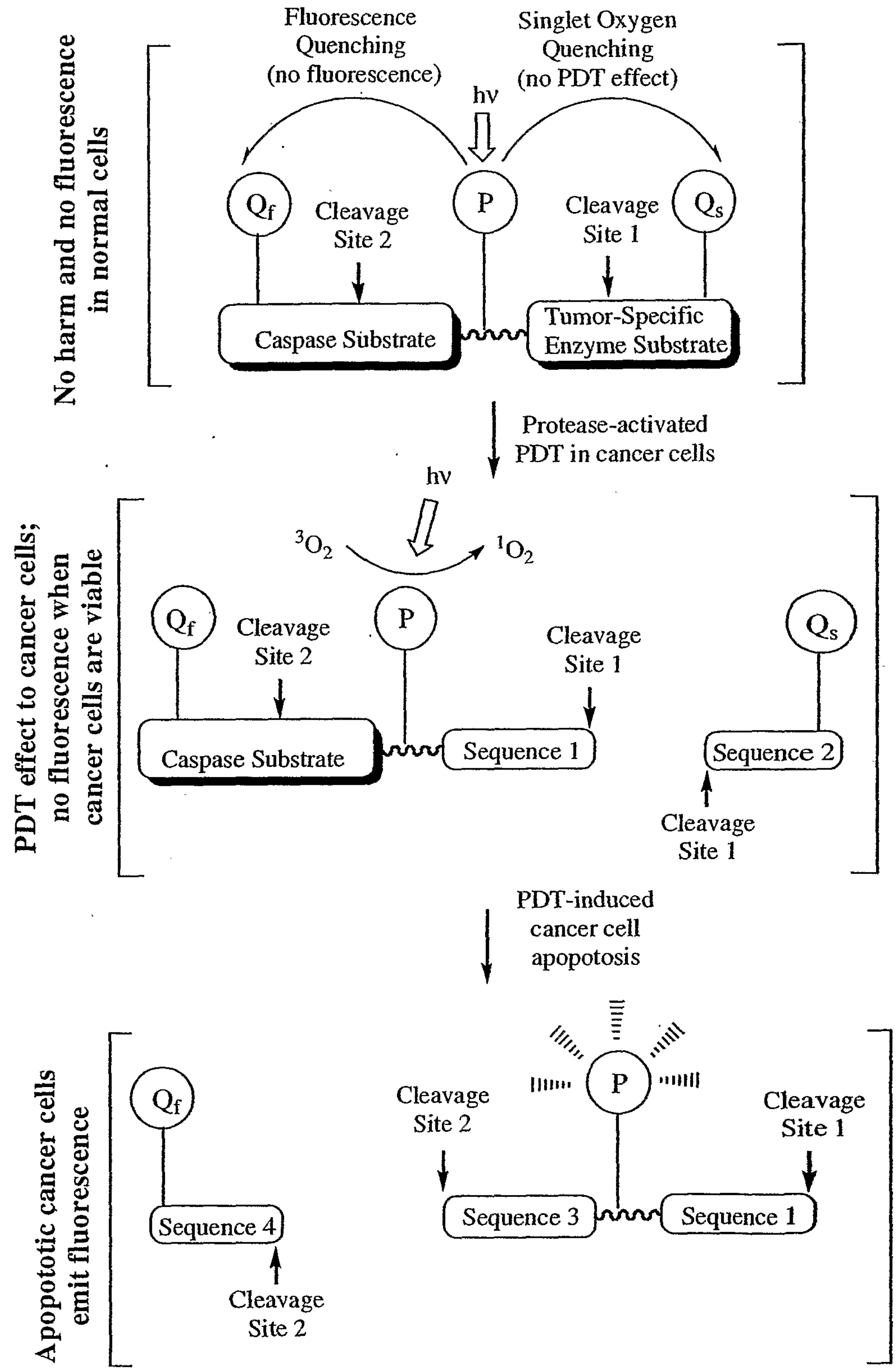

No harm and no fluorescence in normal cells
Fluorescence Quenching (no fluorescence)
Singlet Oxygen Quenching (no PDT effect)
hν
Q_f
P
Q_s
Cleavage Site 2
Cleavage Site 1
Caspase Substrate
Tumor-Specific Enzyme Substrate
Protease-activated PDT in cancer cells
PDT effect to cancer cells; no fluorescence when cancer cells are viable
hν
$^3O_2$
$^1O_2$
Sequence 1
Sequence 2
PDT-induced cancer cell apoptosis
Apoptotic cancer cells emit fluorescence
Sequence 3
Sequence 4

Figure 3

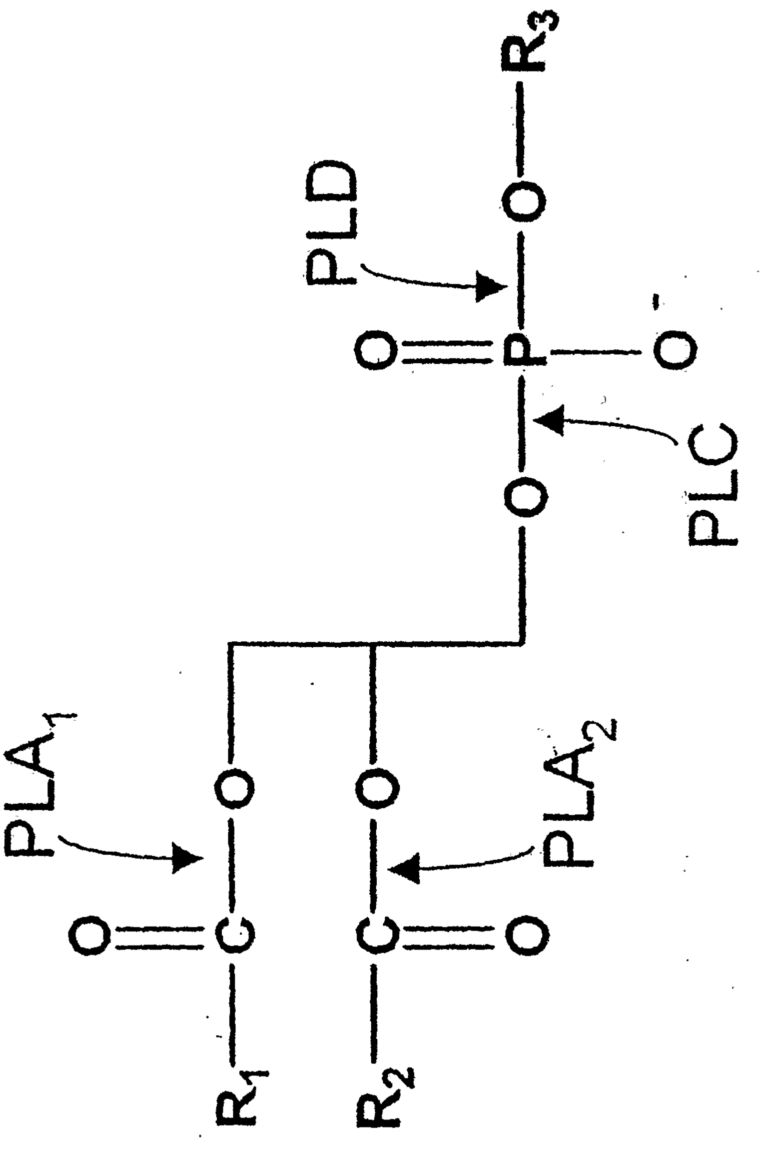


Figure 4

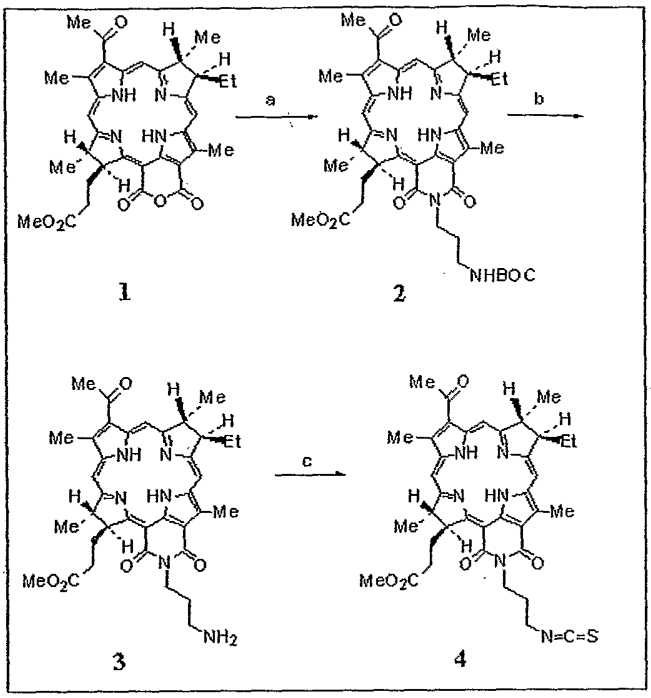


Figure 5

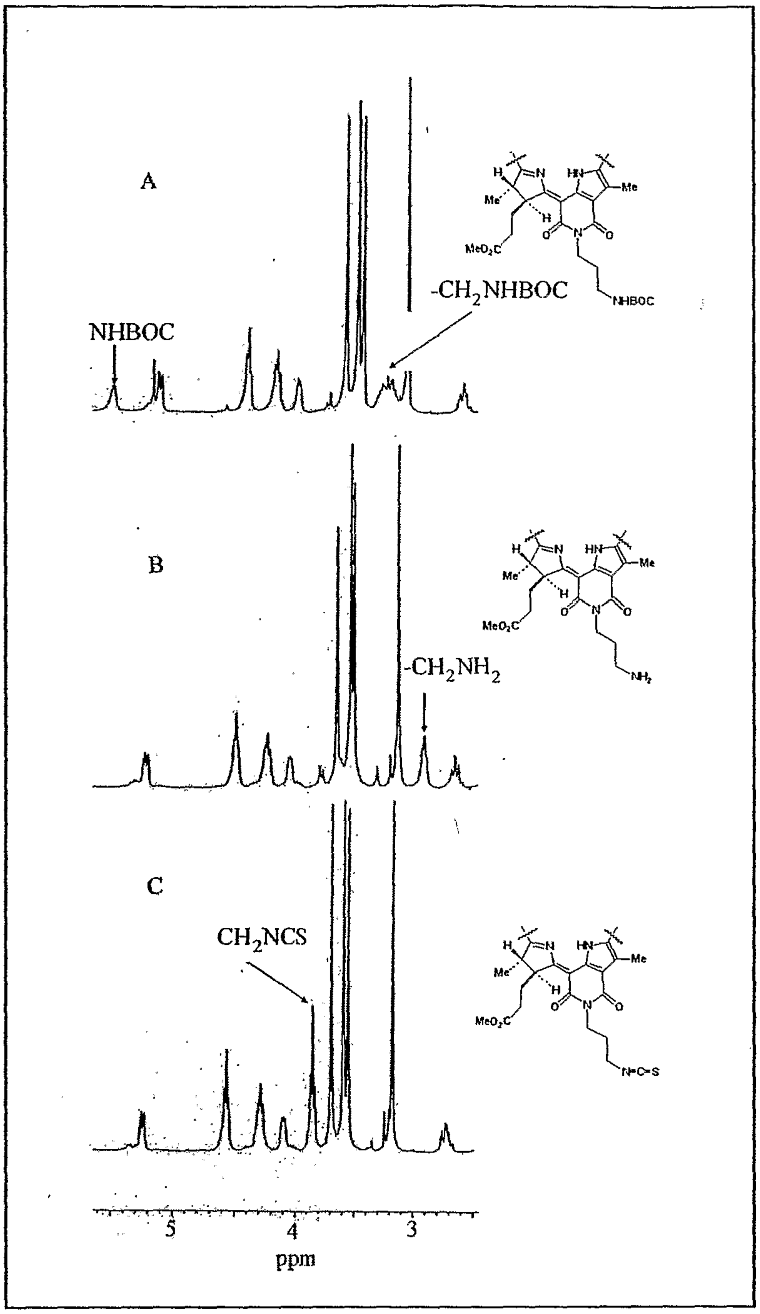
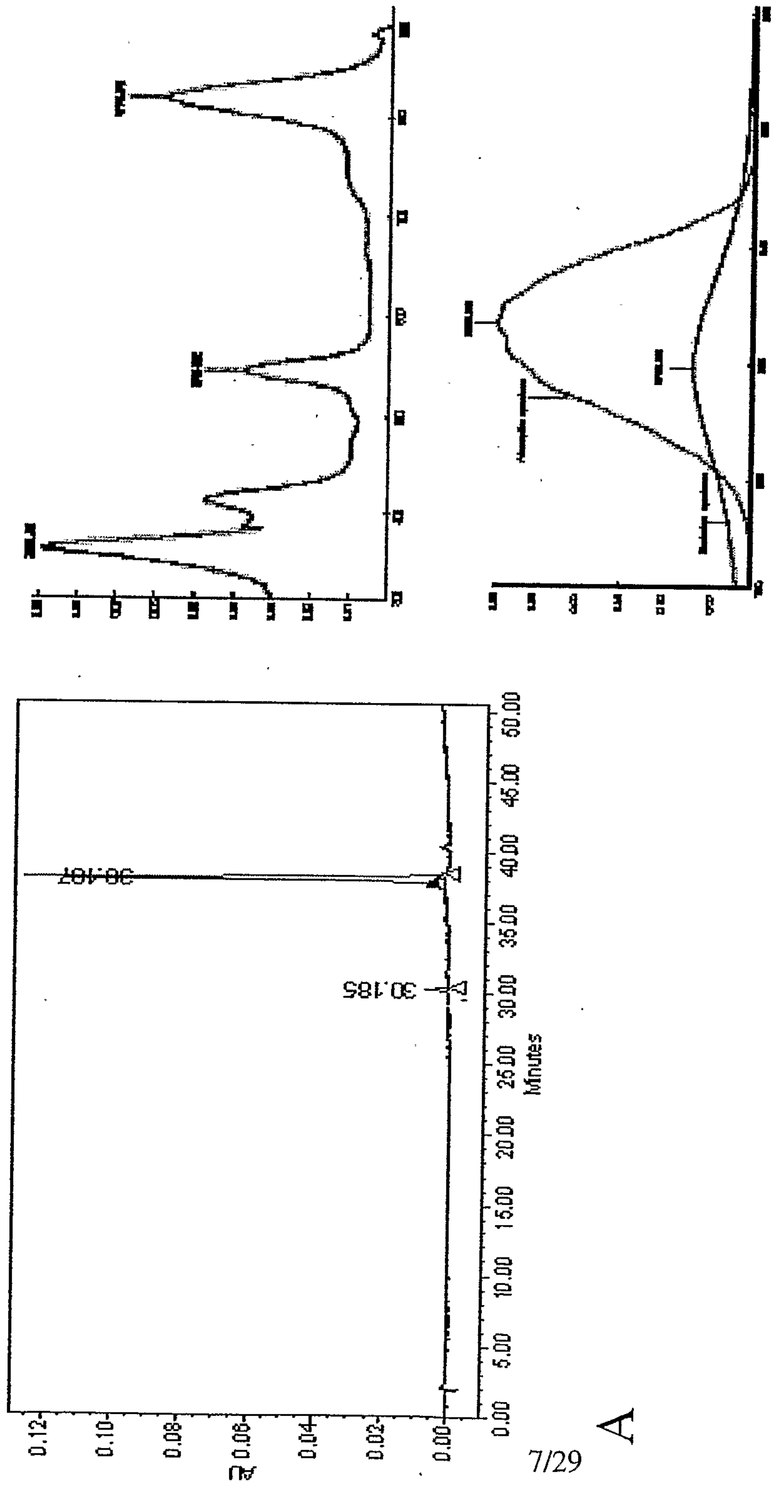


Figure 6


AU
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0.00
5.00
10.00
15.00
20.00
25.00
30.00
35.00
40.00
45.00
50.00
Minutes

A

B

Figure 7

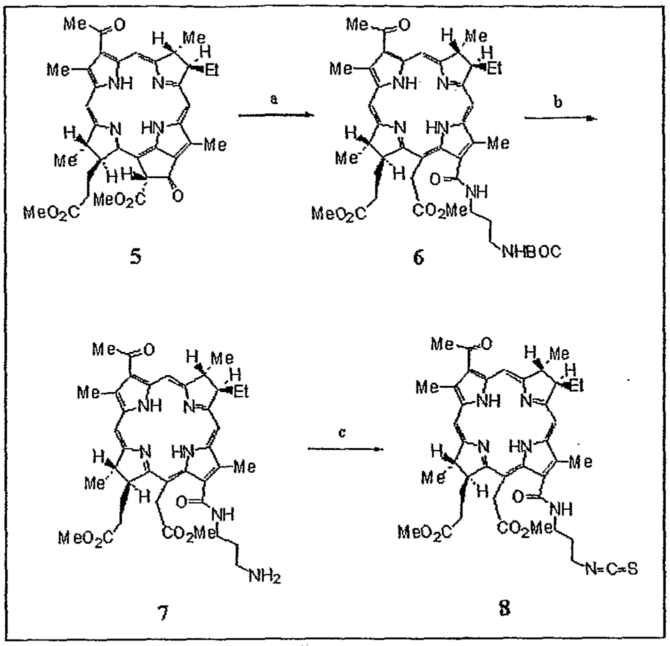


Figure 8

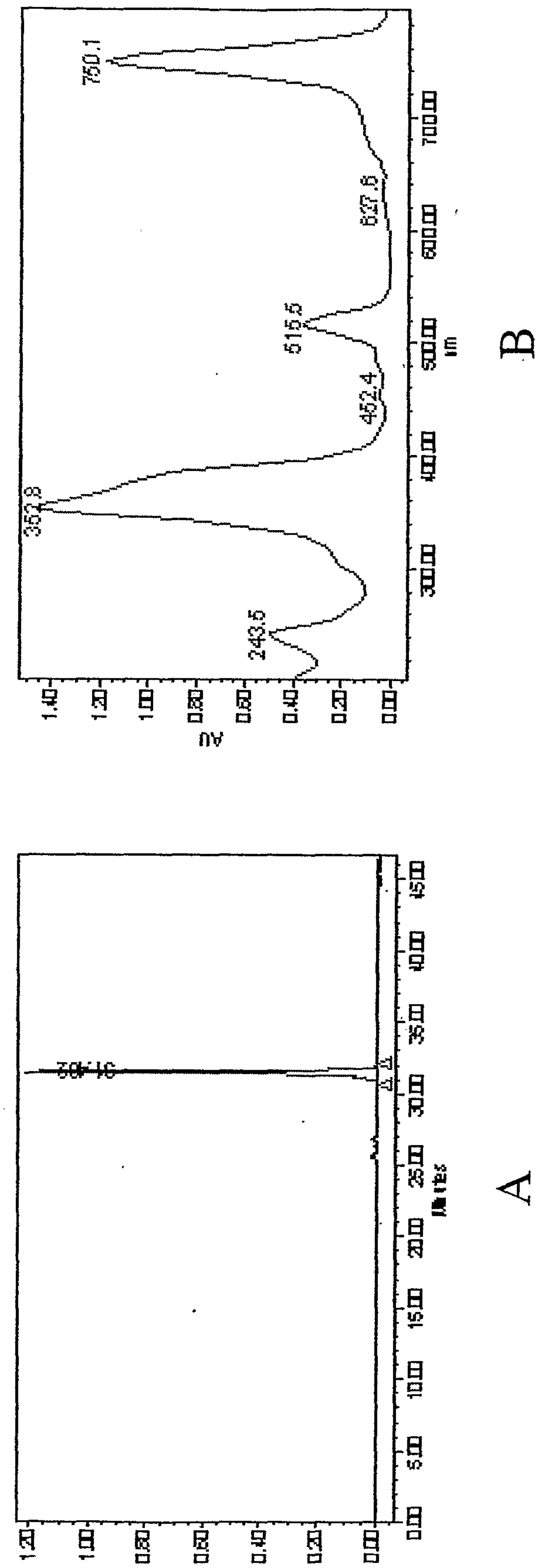


Figure 9

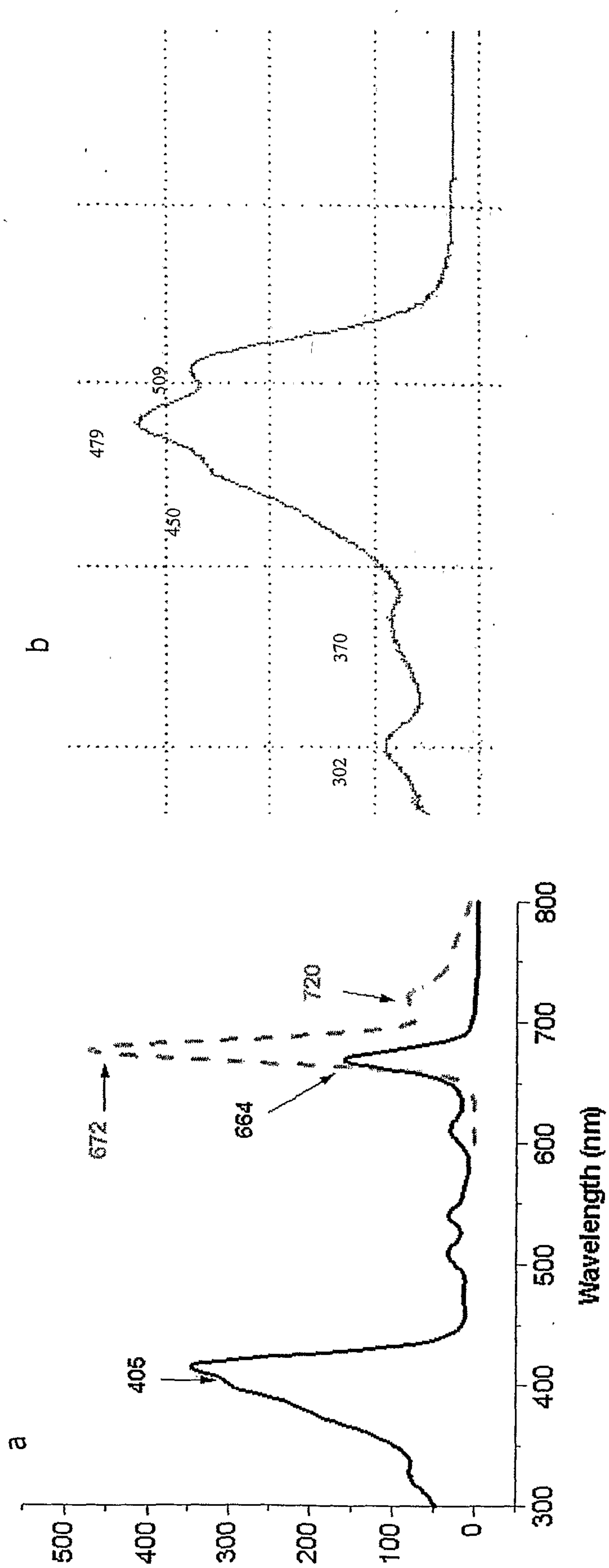

a
405
672
664
720
OD
0
100
200
300
400
500
300
400
500
600
700
800
Wavelength (nm)
b
302
370
450
479
509

Figure 10

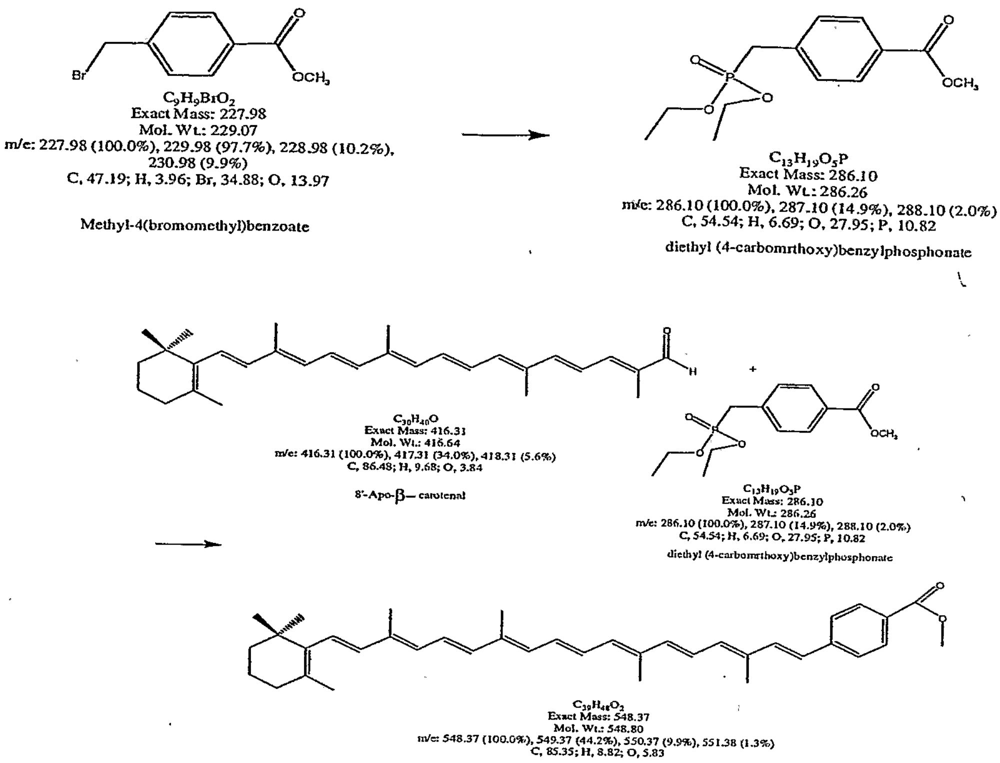
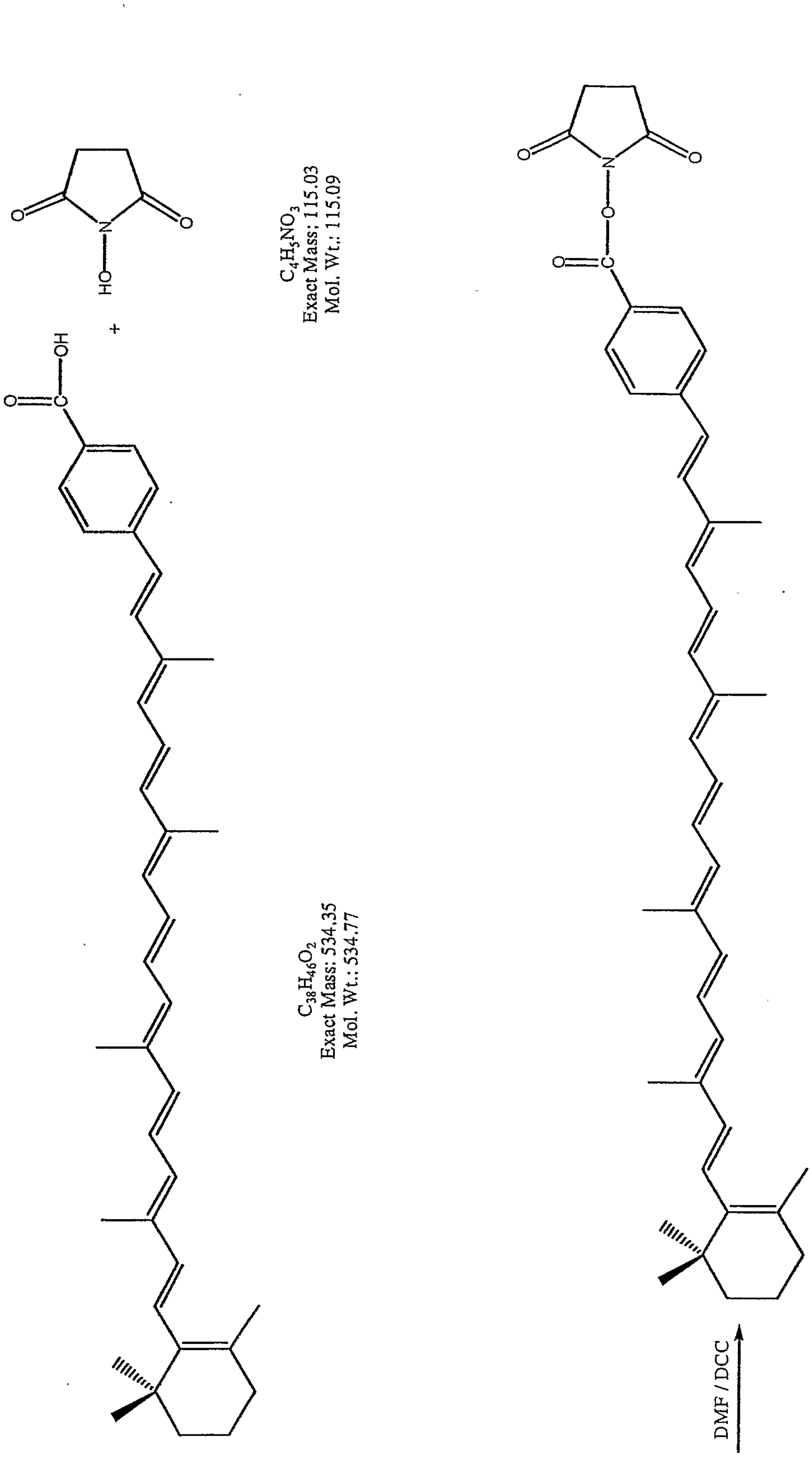

Br
OCH3
C9H9BrO2
Exact Mass: 227.98
Mol. Wt.: 229.07
m/e: 227.98 (100.0%), 229.98 (97.7%), 228.98 (10.2%), 230.98 (9.9%)
C, 47.19; H, 3.96; Br, 34.88; O, 13.97
Methyl-4(bromomethyl)benzoate
C13H19O5P
Exact Mass: 286.10
Mol. Wt.: 286.26
m/e: 286.10 (100.0%), 287.10 (14.9%), 288.10 (2.0%)
C, 54.54; H, 6.69; O, 27.95; P, 10.82
diethyl (4-carbomrthoxy)benzylphosphonate
C30H40O
Exact Mass: 416.31
Mol. Wt.: 416.64
m/e: 416.31 (100.0%), 417.31 (34.0%), 418.31 (5.6%)
C, 86.48; H, 9.68; O, 3.84
8'-Apo-β-carotenal
C39H48O2
Exact Mass: 548.37
Mol. Wt.: 548.80
m/e: 548.37 (100.0%), 549.37 (44.2%), 550.37 (9.9%), 551.38 (1.3%)
C, 85.35; H, 8.82; O, 5.83

Figure 11



HO
N
O
O
+
OH
C
O
$C_4H_5NO_3$
Exact Mass: 115.03
Mol. Wt.: 115.09
$C_{38}H_{46}O_2$
Exact Mass: 534.35
Mol. Wt.: 534.77
DMF / DCC
$C_{42}H_{49}NO_4$
Exact Mass: 631.37
Mol. Wt.: 631.84

Figure 12.

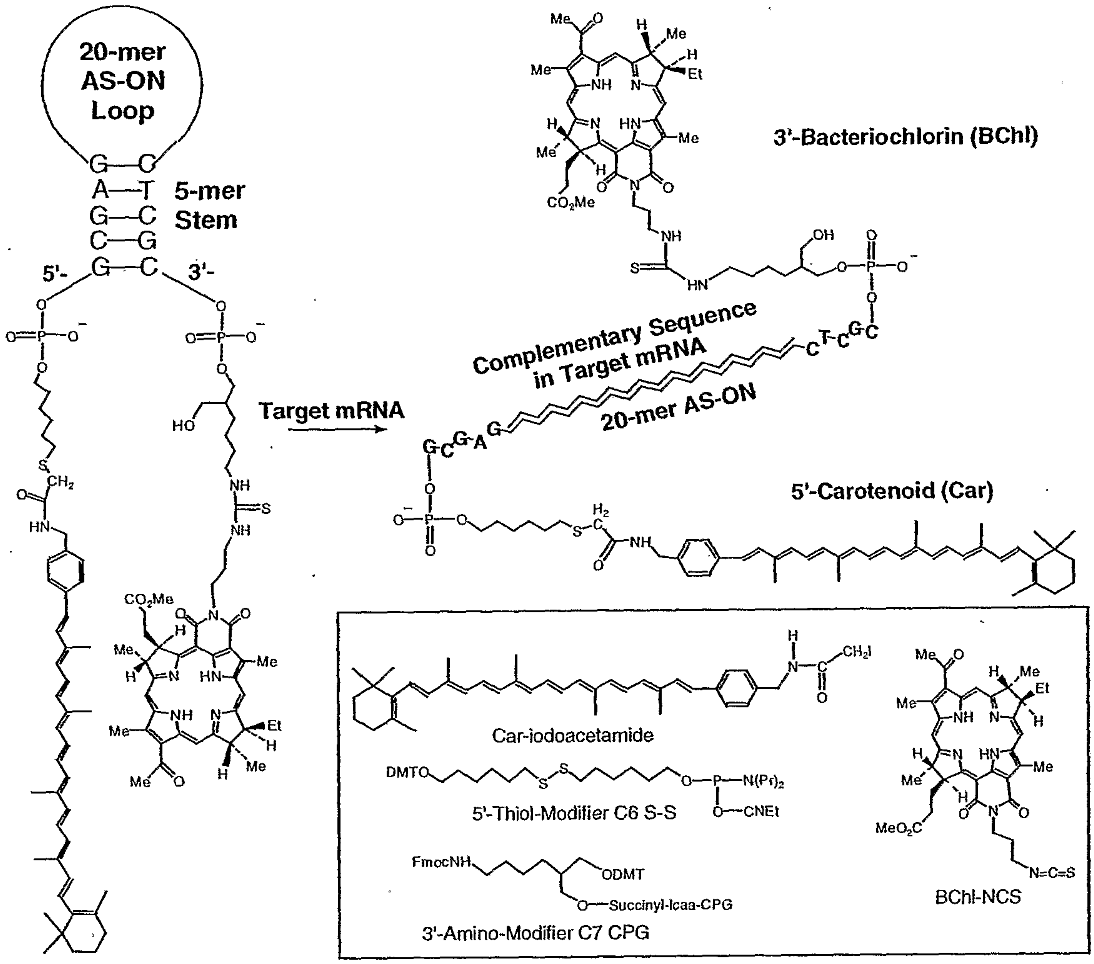

20-mer AS-ON Loop
5-mer Stem
5'-
3'-
Target mRNA
Complementary Sequence in Target mRNA
20-mer AS-ON
3'-Bacteriochlorin (BChl)
5'-Carotenoid (Car)
Car-iodoacetamide
5'-Thiol-Modifier C6 S-S
3'-Amino-Modifier C7 CPG
BChl-NCS

Figure 13

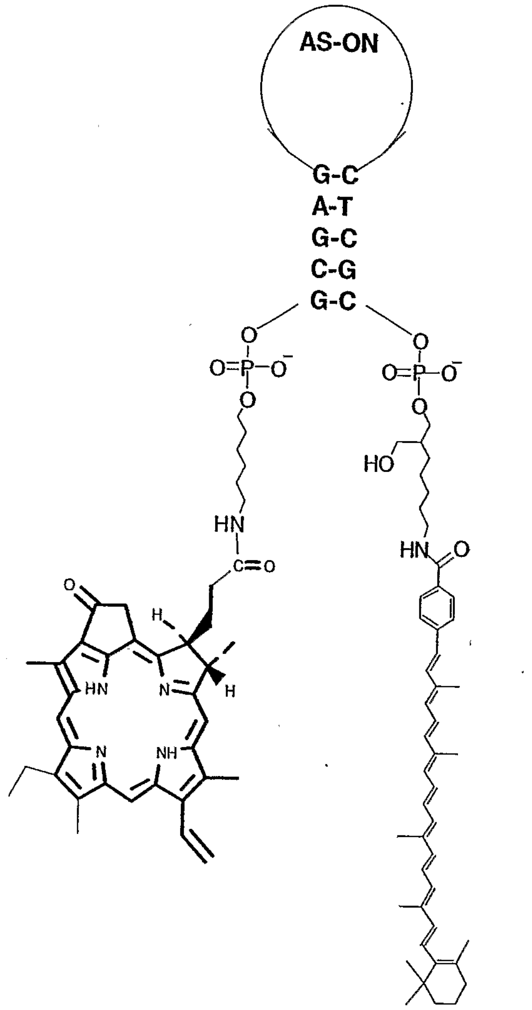


Figure 14

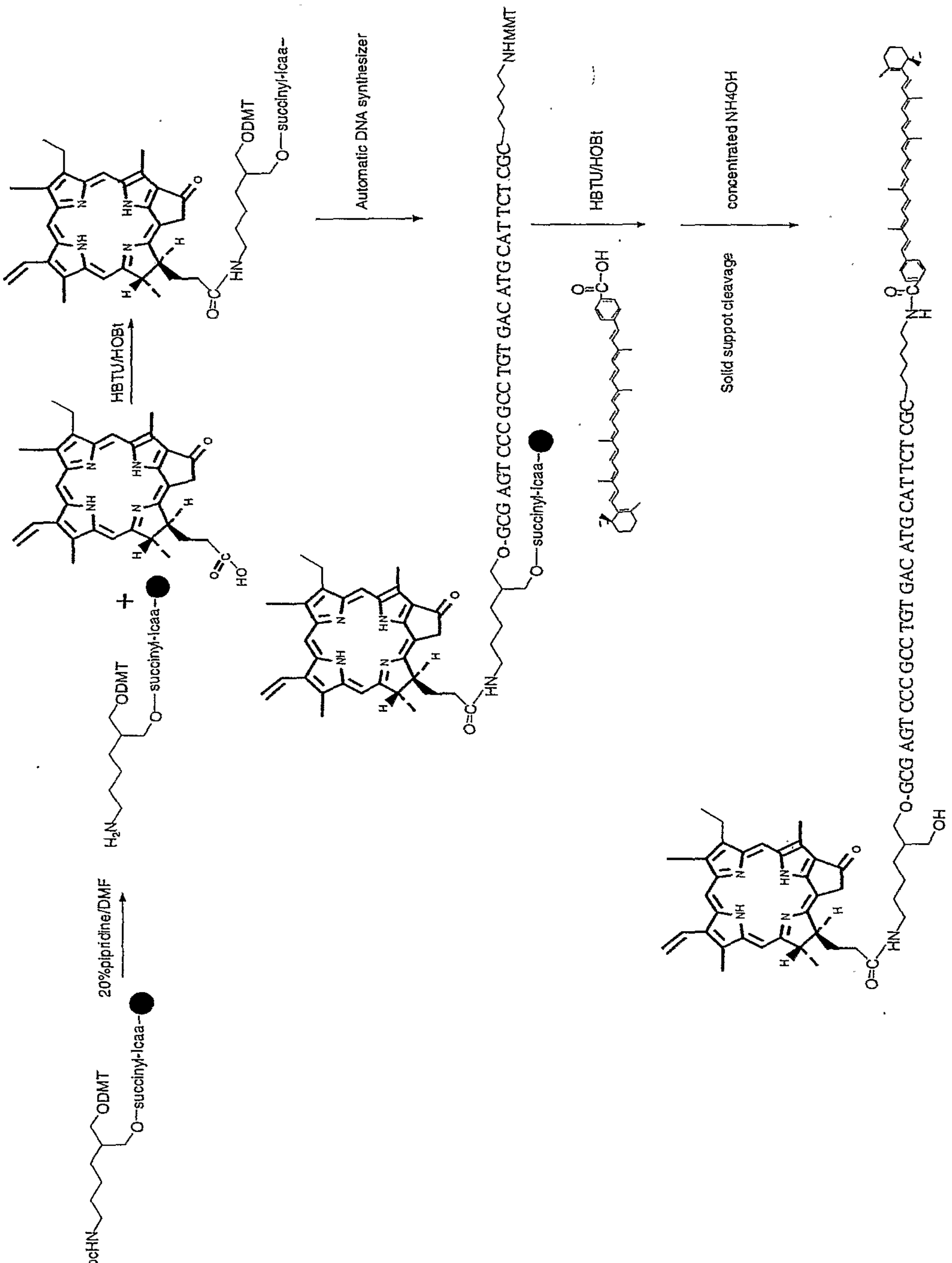

FmocHN
ODMT
O—succinyl-lcaa—
20%pipridine/DMF
H2N
+
HBTU/HOBt
HO
HN
O=C
Automatic DNA synthesizer
O-GCG AGT CCC GCC TGT GAC ATG CAT TCT CGC
NHMMT
HBTU/HOBt
C-OH
concentrated NH4OH
Solid suppot cleavage
OH

Figure 15

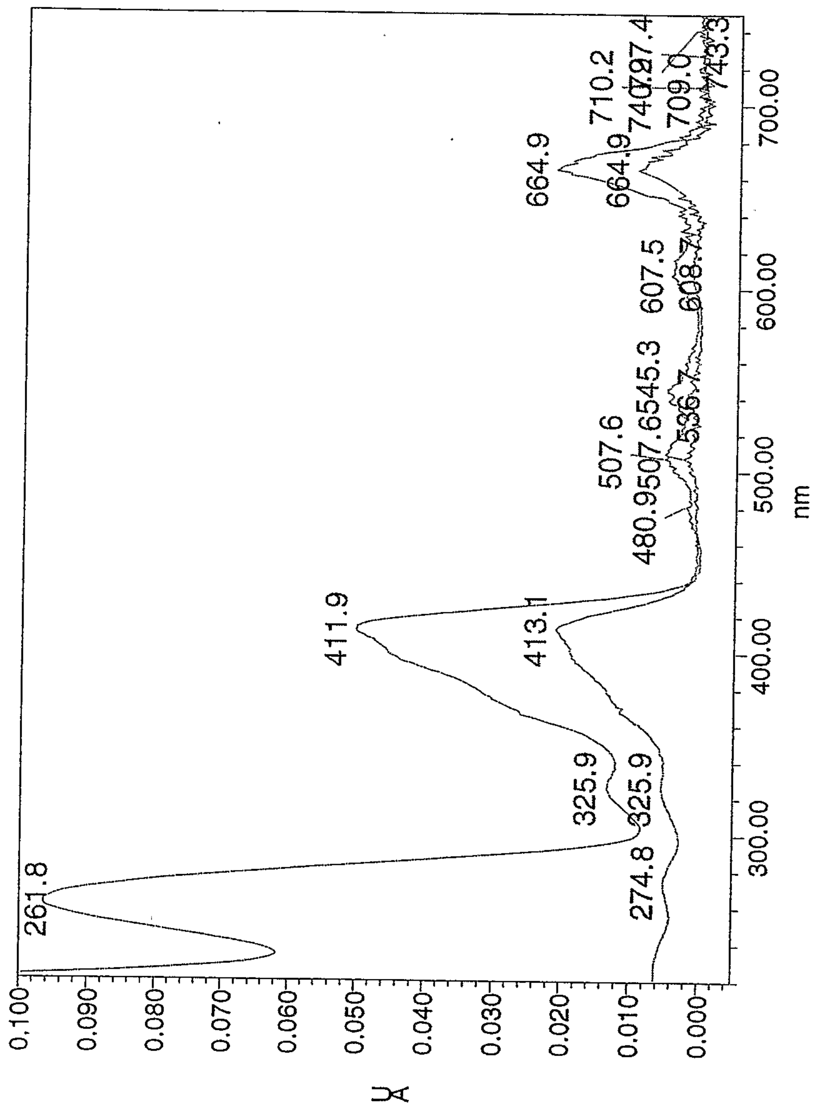

0.100
0.090
0.080
0.070
0.060
0.050
0.040
0.030
0.020
0.010
0.000
300.00
400.00
500.00
600.00
700.00
nm
261.8
274.8
325.9
325.9
411.9
413.1
480.9
507.6
545.3
607.5
664.9
664.9
710.2
709.0

Figure 16.

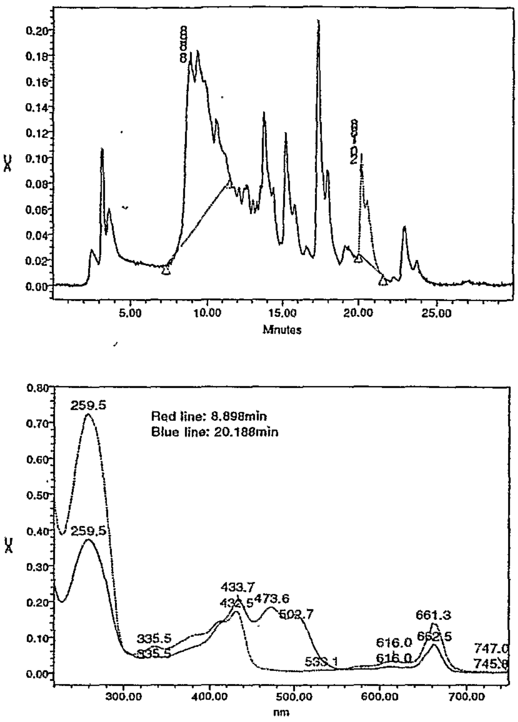


Figure 17

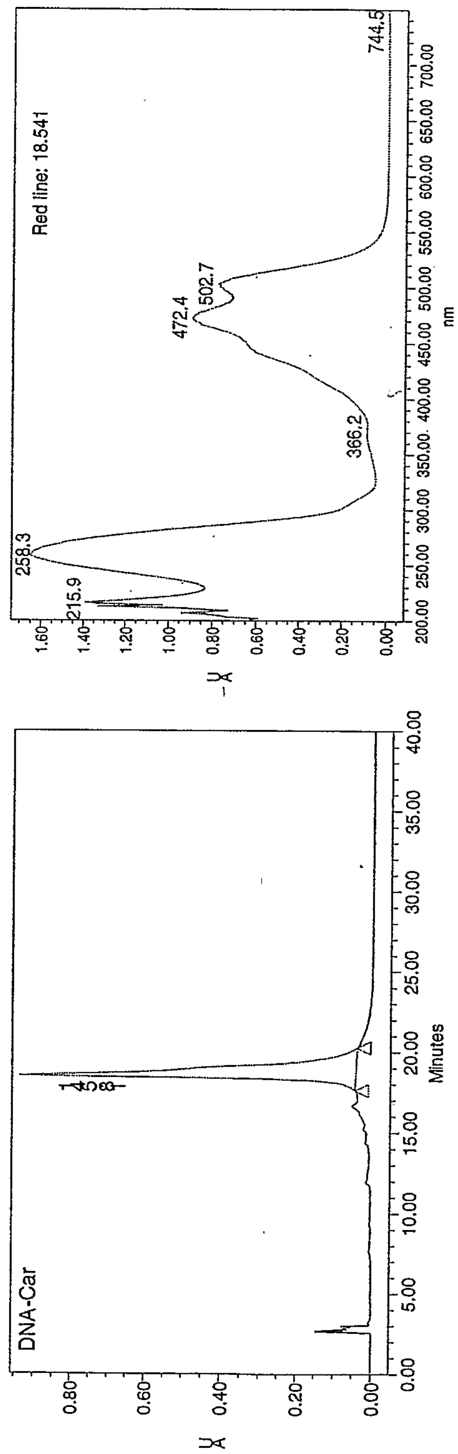

Red line: 18.541
258.3
215.9
366.2
472.4
502.7
744.5
nm
AU
DNA-Car
Minutes
AU

Figure 18.

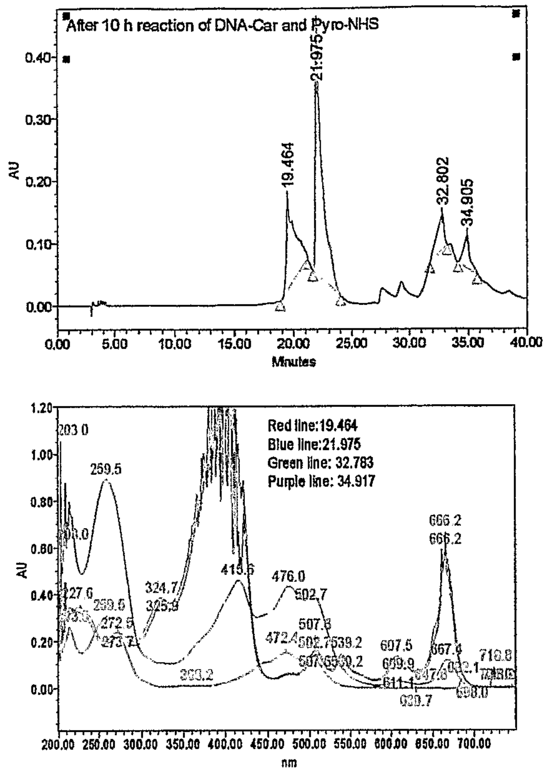

After 10 h reaction of DNA-Car and Pyro-NHS
AU
Minutes
19.464
21.975
32.802
34.905
AU
nm
Red line:19.464
Blue line:21.975
Green line: 32.783
Purple line: 34.917
259.5
666.2
415.6
476.0

Figure 19

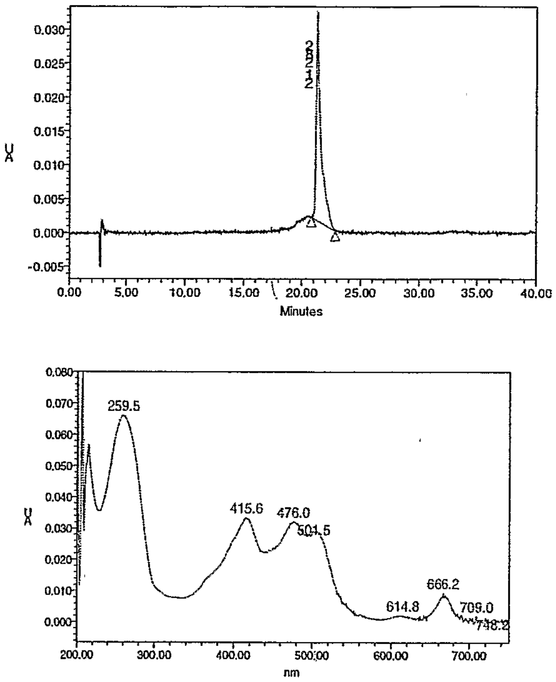

0.030
0.025
0.020
0.015
0.010
0.005
0.000
-0.005
AU
0.00
5.00
10.00
15.00
20.00
25.00
30.00
35.00
40.00
Minutes
0.080
0.070
0.060
0.050
0.040
0.030
0.020
0.010
0.000
AU
259.5
415.6
476.0
501.5
614.8
666.2
709.0
200.00
300.00
400.00
500.00
600.00
700.00
nm

Figure 20

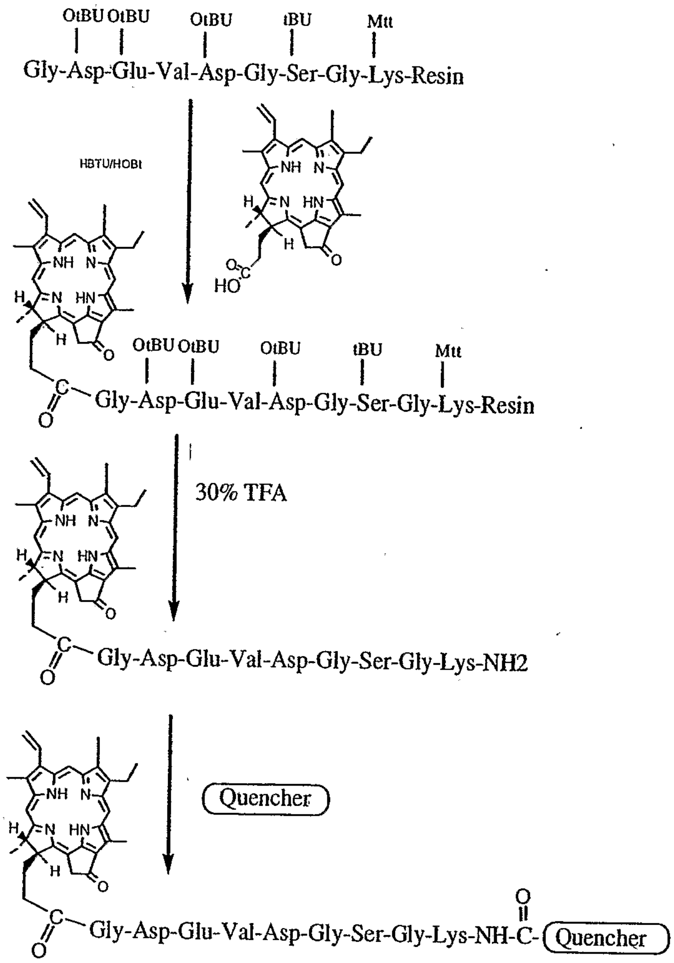

OtBU OtBU OtBU tBU Mtt
Gly-Asp-Glu-Val-Asp-Gly-Ser-Gly-Lys-Resin
HBTU/HOBt
Gly-Asp-Glu-Val-Asp-Gly-Ser-Gly-Lys-Resin
30% TFA
Gly-Asp-Glu-Val-Asp-Gly-Ser-Gly-Lys-NH2
Quencher
Gly-Asp-Glu-Val-Asp-Gly-Ser-Gly-Lys-NH-C-Quencher

Figure 21

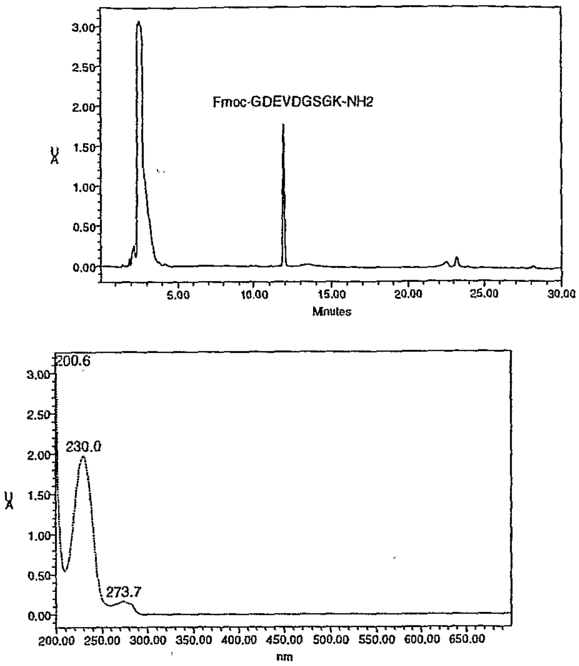


Figure 22

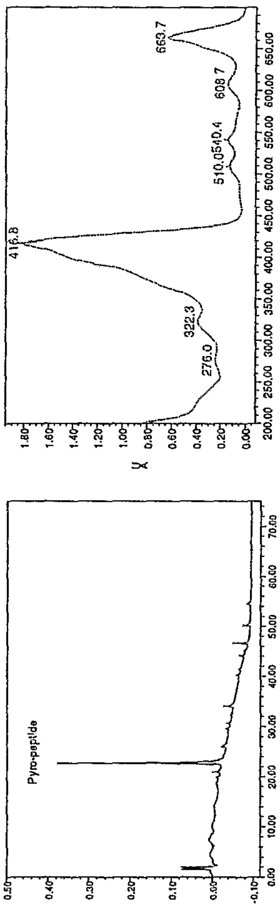


Figure 23.

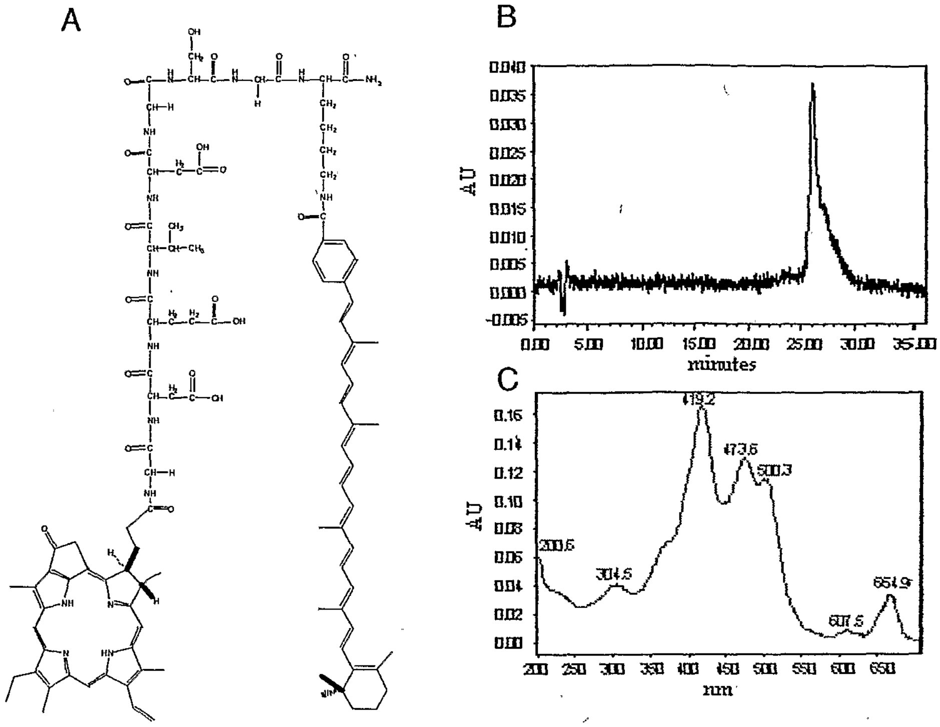


Figure 24

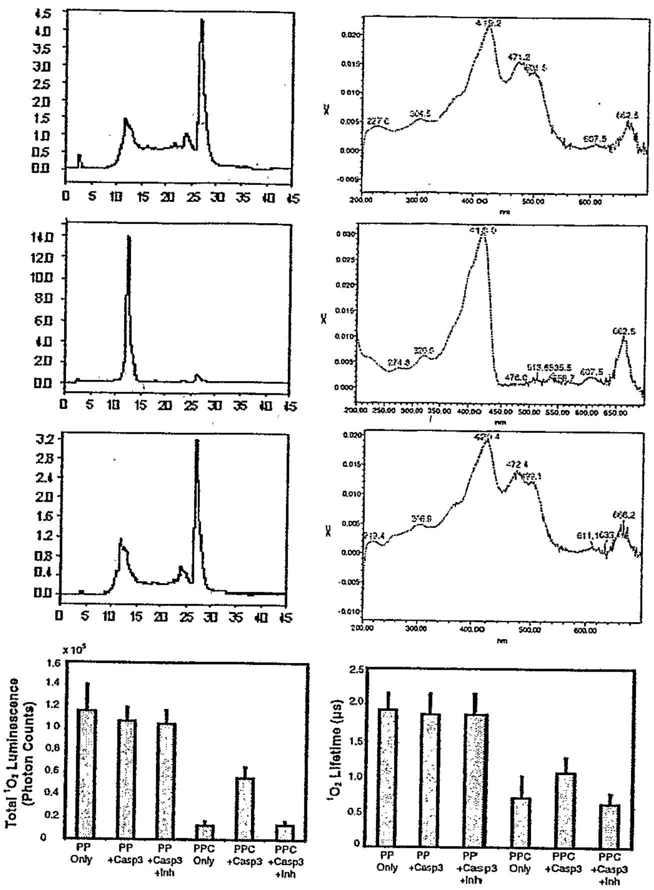


Figure 25

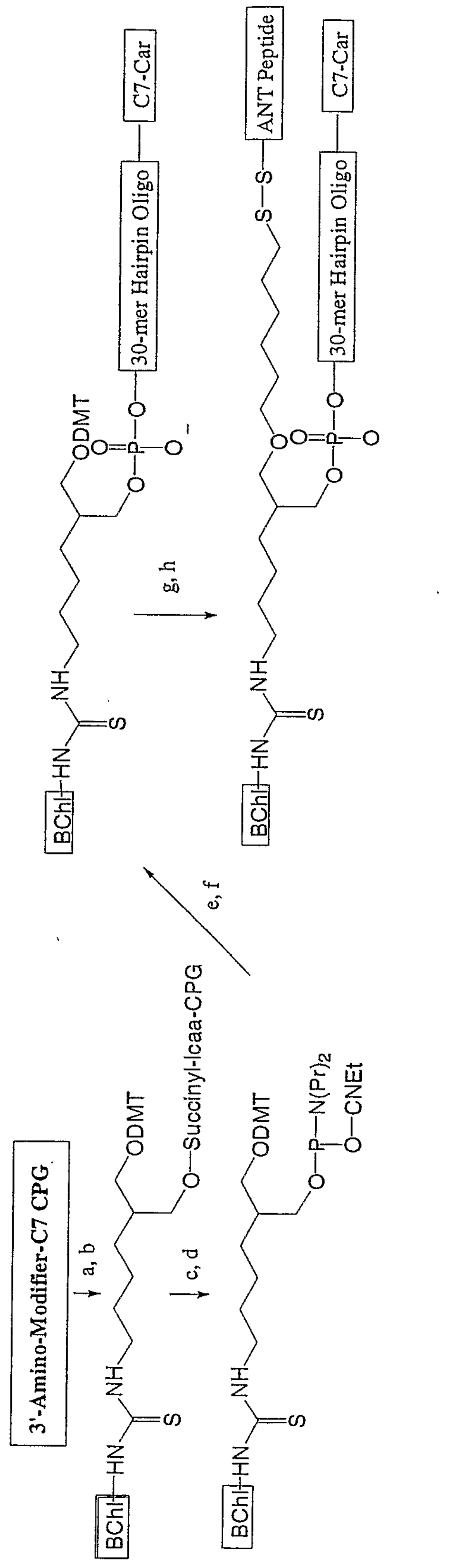

3'-Amino-Modifier-C7 CPG
a, b
c, d
e, f
g, h
BChl
HN
NH
S
ODMT
O—Succinyl-lcaa-CPG
N(Pr)2
O—CNEt
30-mer Hairpin Oligo
C7-Car
ANT Peptide
S—S

Figure 26

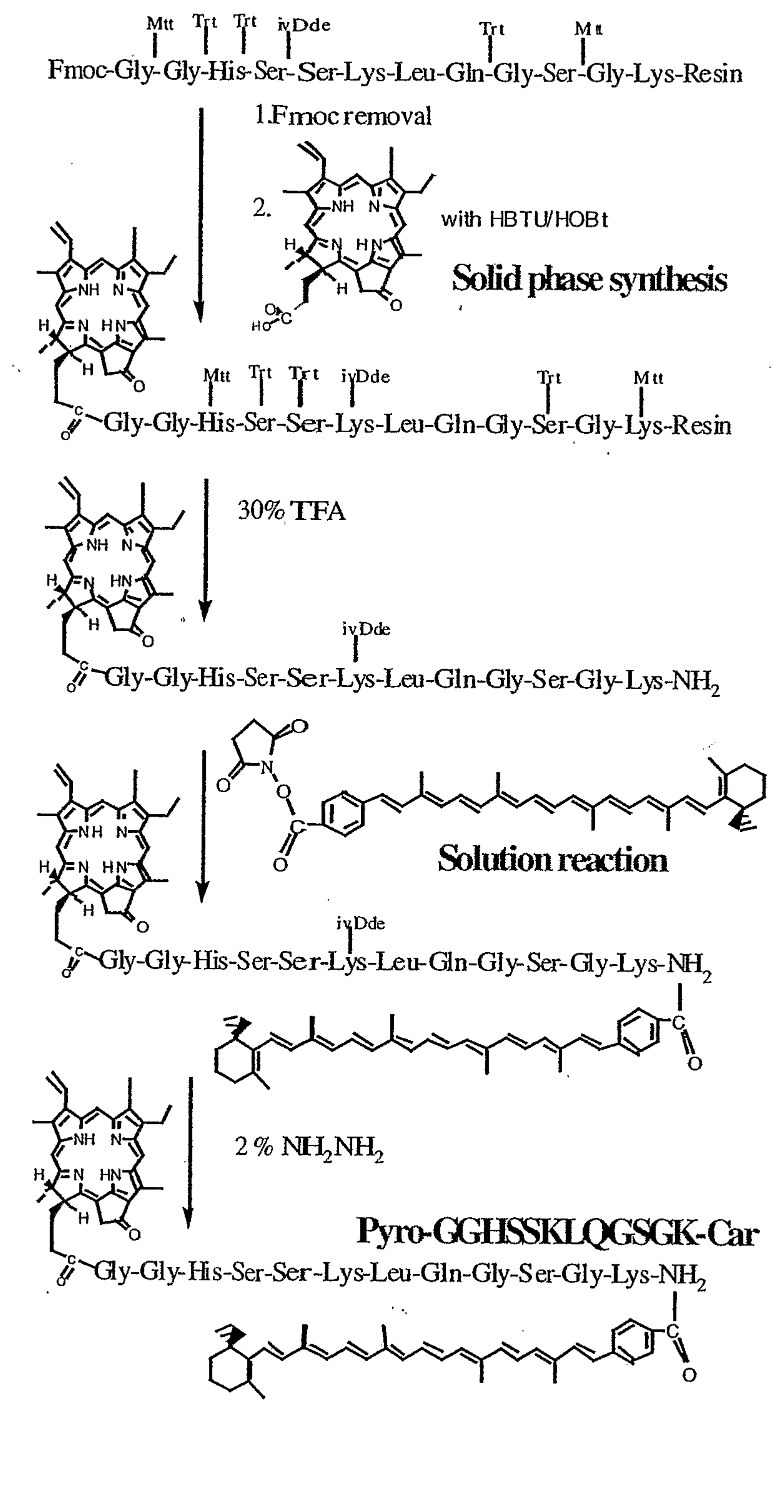

Fmoc-Gly-Gly-His-Ser-Ser-Lys-Leu-Gln-Gly-Ser-Gly-Lys-Resin
Mtt
Trt
Trt
ivDde
Trt
Mtt
1.Fmoc removal
2.
with HBTU/HOBt
Solid phase synthesis
Gly-Gly-His-Ser-Ser-Lys-Leu-Gln-Gly-Ser-Gly-Lys-Resin
30% TFA
Gly-Gly-His-Ser-Ser-Lys-Leu-Gln-Gly-Ser-Gly-Lys-NH2
Solution reaction
2 % NH2NH2
Pyro-GGHSSKLQGSGK-Car

Figure 27

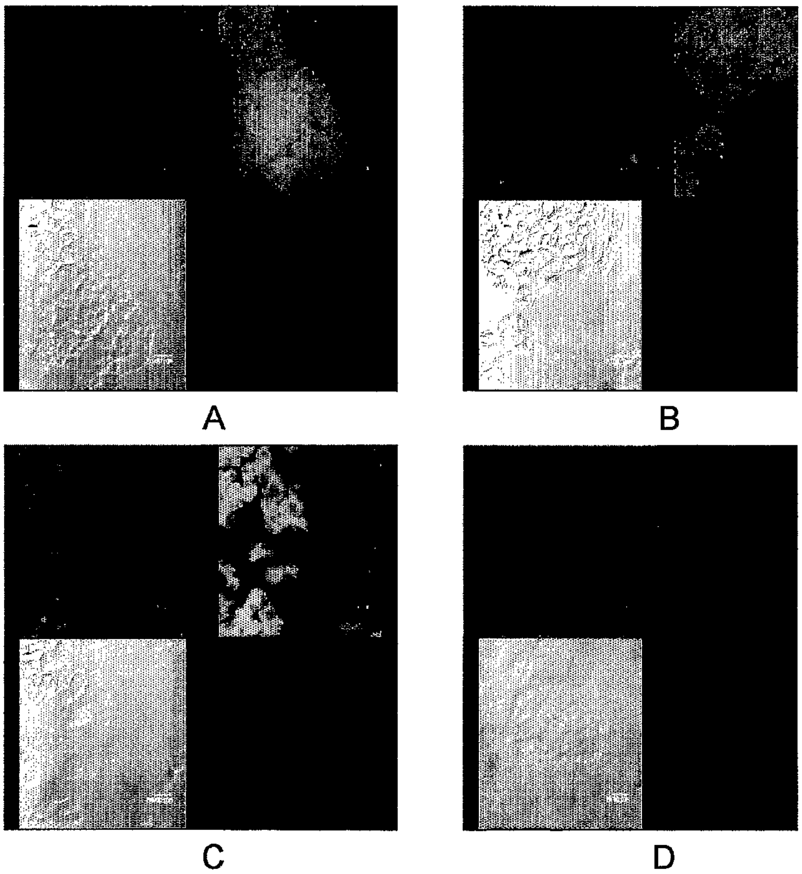


Figure 28

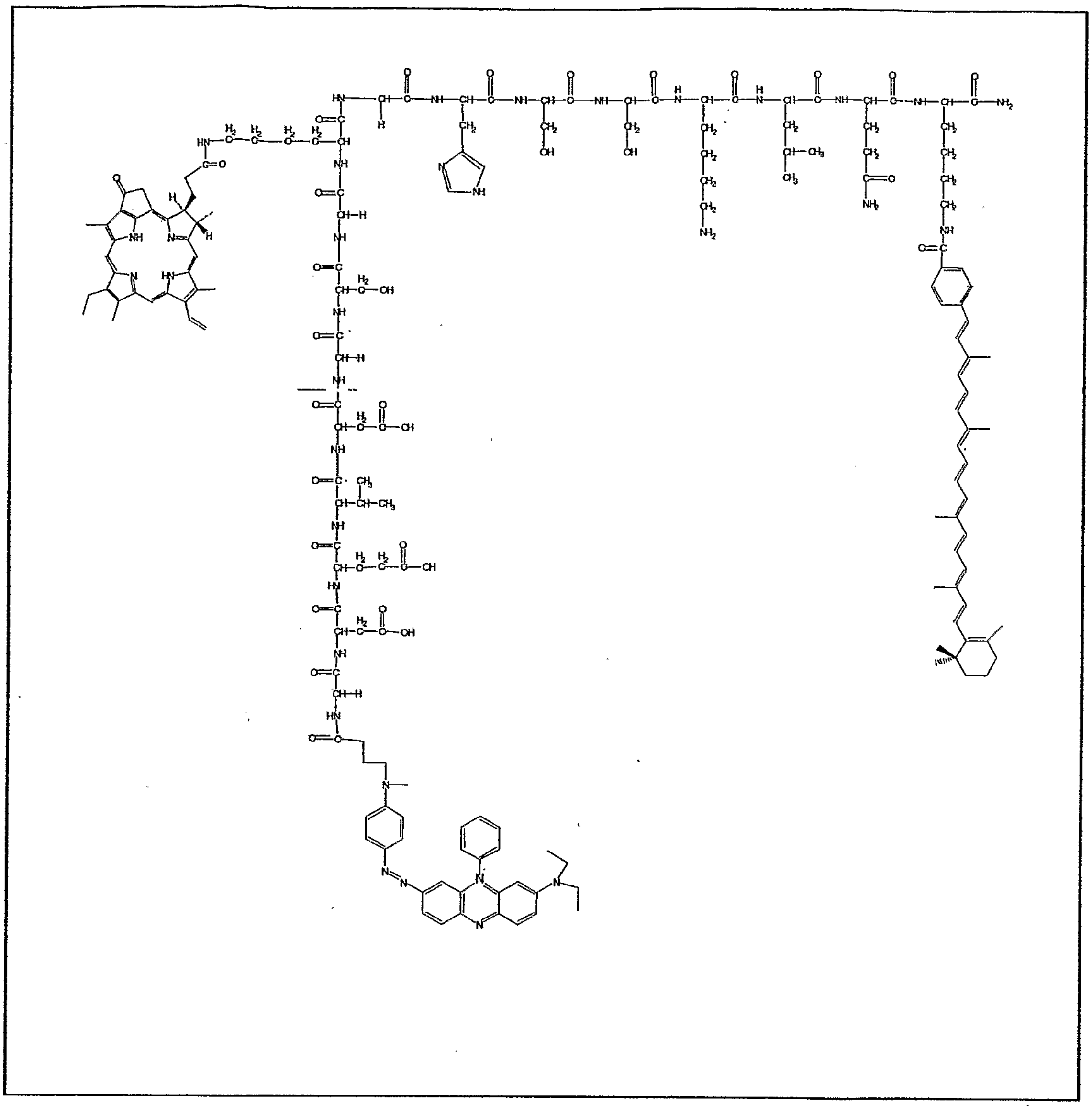


Figure 29