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(54) Title: PEPTIDE PRODRUGS

(57) Abstract: The present invention relates to the field of prodrugs, and more particularly prodrugs that are intended for the treatment and/or diagnosis of tumors and/or inflammatory reactions.

PEPTIDE PRODRUGS

BACKGROUND OF THE INVENTION

The present invention relates to the field of prodrugs, and more particularly prodrugs that are intended for the treatment and/or diagnosis of tumors and/or inflammatory reactions.

Prodrugs are pharmacologically inactive molecules that are transformed *in vivo* into active therapeutic agents after certain chemical or enzymatic modifications of their structure. Prodrugs are intended to release the therapeutic agent at a target site or tissue rather than in the circulatory structure or in a non-target tissue. They also make it possible to increase *in vivo* the therapeutic index of therapeutic agents such as anti-tumor agents like anthracyclines (such as doxorubicin) and vinca alkaloids or anti-tumor agents that have anti-inflammatory effects like methotrexate. Thus, several prodrugs have been developed to date for the purpose of obtaining high action specificity, a reduced toxicity and an improved activity, for example through improved stability in the blood and/or the plasma.

Prodrugs comprising a stabilizing or masking group covalently linked to a therapeutic agent via an oligopeptide cleavable by an enzyme, specifically by a peptidase, have been described in the prior art. See, for example, U.S. Pat No US 4,703,107 [1] and US 5,618,790 [2].

International patent application No. WO 96/05863 [3] describes peptide prodrugs of therapeutic agents, selectively cleavable by factors secreted by target cells so as to enable the therapeutic agent to penetrate into said target cells. Such prodrugs have a stabilizing group, for example a beta-alanyl or succinyl moiety, ensuring that the prodrug is stable in serum and circulating blood. *In vivo* toxicity and activity studies with the prodrug beta-alanyl-leucyl-alanyl-leucyl-doxorubicin or succinyl beta-alanyl-leucyl-alanyl-leucyl-doxorubicin according to WO 96/05863 [3] showed a reduction in toxicity and a more important inhibition of tumor growth compared to doxorubicin (Dubois *et al.*, 2002, Cancer Res., 62(8):2327-31 [4] ; Trouet *et al.*, 2001, Cancer Res., 61(7):2843-46 [5]).

International patent application No. WO 00/33888 [6] discloses the addition of a group that masks the positive charge of beta-alanine to the prodrug beta-alanyl-leucyl-

alanyl-leucyl-doxorubicin described above so as to reduce its toxicity. This masking group can be, for example, a succinic acid.

Masking groups have also been described in PCT application No. WO 00/71571 [7] aiming to increase the hydrophilicity of peptide prodrugs and thus enhance their solubility in aqueous media. For example, disclosed but not exemplified masking groups are cyclic hydrocarbon radicals having 3 to 8 carbon atoms which may optionally be substituted by one or several hydroxyl, amino, C_1-C_6-alkyl-amino, di- C_1-C_6-alkyl-amino, C_1-C_6-alkyl, C_1-C_6-alkyloxy, thiol, C_1-C_6-alkyl-thio, =O, =NH, -CHO, -COOH, $-COOCH_3$, $-COOCH_2CH_3$, $-CONH_2$, $-NHC(=NH)NH_2$, or halogen substituents, which may be identical to one another or different.

The present invention aims to provide peptide prodrugs of a therapeutic agent or marker, which improve solubility of the therapeutic agent or marker, reduce its toxic or non-desirable side-effects, improve its therapeutic index, improve its toxicological profile and/or modify its tissue distribution and/or metabolism, as compared to known peptide prodrugs.

BRIEF SUMMARY OF THE INVENTION

The present invention relates to the modification of a peptide prodrug with a 1,2,3,4-cyclobutanetetracarboxylic acid (hereinafter designated by TA) derived moiety for enhancing the effects of said peptide prodrug. The present invention further discloses novel peptide prodrugs presenting said TA derived moiety, compositions comprising these prodrugs and uses thereof.

According to a first aspect, the invention relates to a compound (peptide prodrug) comprising a therapeutic agent linked directly or indirectly to a cleavable oligopeptide which, in turn, is linked directly or indirectly to a TA derived moiety. The compounds of the present invention provide numerous benefits including, in particular, improvement of the therapeutic index and/or the solubility of the therapeutic agent.

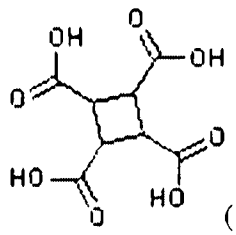
The invention also relates to pharmaceutical compositions comprising such peptide prodrugs and therapeutic uses thereof, in particular for the treatment of various types of diseases, including cancers.

The invention also relates to the use of at least one compound defined herein below for the preparation of a medicament for treating a number of disorders.

DETAILED DESCRIPTION OF THE INVENTION

In one aspect, the invention provides a compound comprising a therapeutic agent or marker linked directly or indirectly to an oligopeptide which, in turn, is linked directly or indirectly to a 1,2,3,4-cyclobutanetetracarboxylic acid derived moiety.

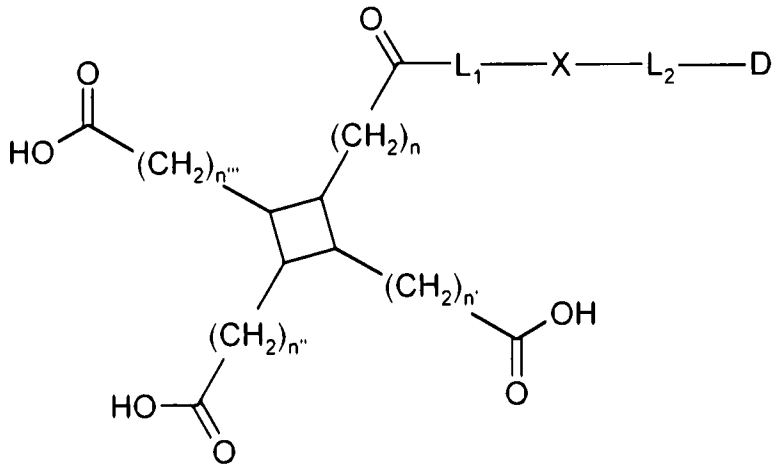
1,2,3,4-cyclobutanetetracarboxylic acid (TA) is a well-known and commercially available compound. It has the following formula, $C_8H_8O_8$, or formula (I):



(I)

The present inventors have shown that modification of a peptide prodrug with TA leads to several beneficial advantages over the peptide prodrugs of the prior art, including the reduction of the toxicity of the peptide prodrug of the invention compared to the free drug, the increase of its efficacy and/or improved solubility.

Accordingly, it is an aspect of the invention to provide peptide prodrugs which are compounds of formula (II)



(II)

wherein

L_1 and L_2 are independently a covalent bond or a linking moiety, said linking moiety should include at least two organic functional groups, one organic functional group that can bind to the 1,2,3,4-cyclobutanetetracarboxylic acid and a second organic functional group that allows binding to the oligopeptide moiety (L1) or one functional group that can bind to the oligopeptide moiety and a second organic functional group that allows binding to D (L2) ;

n, n', n'' and n''' independently represent an integer comprised between 0 and 10;

X is a cleavable oligopeptide moiety, said moiety being cleavable by at least one specific and/or selective peptidase that is present in the extracellular environment of target cells;

D is a therapeutic agent or marker ;

or the pharmaceutically acceptable salts thereof.

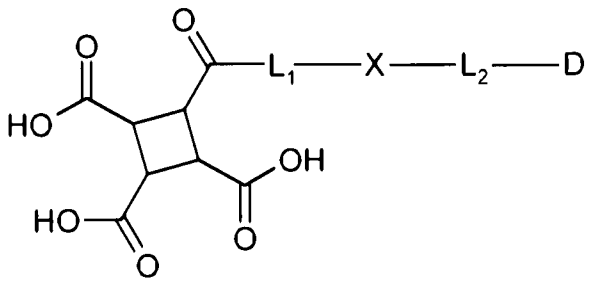
Advantageously, modification of a therapeutic agent such as to obtain a compound according to the invention can reduce some of the side effects of said therapeutic agent. Furthermore, peptide prodrugs of the invention present enhanced stability/efficiency and reduced toxicity of the therapeutic agent compared to known peptide prodrugs of the prior art.

Advantageously, the TA derived moiety in the compounds of formula (II) blocks the cleavage of the compounds of the invention by enzymes (*e.g.* peptidases, in particular exopeptidases) *in vivo* in the blood (or the circulatory structure) or in the extracellular space of non-target tissue. The present inventors have demonstrated that addition of a TA derived moiety results in an enhancement of the therapeutic efficiency compared to known peptide prodrugs and a reduction of the toxicity of the prodrug in comparison with the free drug. Additionally, this TA derived moiety provides preferable charge and/or other physical characteristics to the compounds of the invention. It also improves the pharmacokinetic properties of the prodrug compared to that observed with the prodrugs in the prior art. This result is obtained in particular when the renal elimination of the compounds of the invention or when the degradation or biotransformation by the hepatic

metabolism of the compounds of the invention are reduced. Furthermore, the TA derived moiety can improve inactivation of the prodrug, increase prodrug stability *in vitro* and *in vivo*, improve the solubility of drug compounds and/or increase the antitumoural activity.

Addition of a TA derived moiety to a peptide prodrug according to the present invention thus provides a potent way to enhance the beneficial effects of said peptide prodrug.

In the compounds according to the invention, n, n', n'' and n''' independently represent an integer comprised between 0 and 10, preferably comprised between 0 and 5 (i.e. n, n', n'' and n''' independently represent 0, 1, 2, 3, 4 or 5). In one embodiment of the invention n = n' = n'' = n'''. In a particular embodiment of the invention, in the following formula (III), n = n' = n'' = n''' = 0 :



(III)

The compounds of the invention comprise a cleavable oligopeptide moiety directly or indirectly linked to the TA derived moiety. The cleavage of the oligopeptide moiety allows for activation of the therapeutic agent or marker by releasing the agent or marker from the remaining part of the prodrug in a specific location, *i.e.* in the environment of a target tissue or cells.

According to the present invention, a cleavable oligopeptide is an oligopeptide that can be cleaved selectively by a target tissue- or target cell-associated enzyme (or peptidase), i.e. an oligopeptide which can be cleaved by an enzyme that is present in the environment of the target cells and which is only slightly, and preferably not at all, degraded in the circulatory structure or close to non-target cells. The expression "in the environment of the target cells" means that the enzymatic activity is present extracellularly close to or on the target cells.

It should be noted that even if the cleavage is not carried out on or only close to the target cells, the fact that the cleavage is carried out preferentially (or in large part or in the majority of the cases) close to or on the target cells makes this cleavage selective. For example, the cleavage is qualified selective when the enzyme is in a larger concentration close to or at the target cells relative to other parts of the organism.

The amino acid sequence of the cleavable oligopeptide moiety of the invention is selected for susceptibility to cleavage by at least one enzyme which is present in the environment of the target cells and which is only slightly, and preferably not at all, degraded in the circulatory structure or close to non-target cells.

"Target cells" are defined more particularly as cells that are involved in a pathological condition that are preferred targets for therapeutic or diagnostic activity. "Target tissue" comprises target cells themselves (*e.g.*, tumor cells) but also any cell in the environment of the target cells, such as endothelial and stromal cells. These target cells are preferably selected from the following non limiting group: primary or secondary (metastases) tumor cells, stromal cells of the environment of primary or secondary tumors, neoangiogenic endothelial cells of tumors, macrophages, monocytes, polymorphonuclear leukocytes, lymphocytes, polynuclear cells infiltrating the tumors and the tumor metastases and cells participating in inflammatory reactions, especially associated with rheumatic diseases (e.g. macrophages, neutrophils, osteoblasts, osteocytes and monocytes).

The sequence of the cleavable oligopeptide moiety of the compounds of the invention is selected in accordance to the specific enzymes present in the environment of the target cells or tissue to treat with the therapeutic agent or to monitor with the marker comprised of the said compound of the invention. Indeed, as specified above, the cleavable oligopeptide moieties of the compounds of the invention are characterized by their susceptibility to cleavage by such target tissue- or target cell-associated enzymes.

A "target tissue- or cell-associated enzyme" is defined as a membrane enzyme or an enzyme that is released by the target cells in the extracellular medium surrounding these target cells. Target tissue-associated enzymes according to the invention are preferably peptidases, endopeptidases and lysosomal enzymes (enzymes located within lysosomes).

As indicated above, the compounds of the invention comprise X which is a cleavable oligopeptide moiety cleavable by at least one specific and/or selective peptidase that is present in the extracellular environment of target cells.

According to one embodiment of the invention, the cleavable oligopeptide moiety is cleavable by at least one specific and/or selective peptidase that is present in the extracellular environment of one or more cells that are selected from the group of: tumor cells, stromal cells of tumors, neoangiogenic endothelial cells of tumors and tumor metastases, macrophages, monocytes, polymorphonuclear leukocytes or lymphocytes that infiltrate tumors and tumor metastases

According to a preferred embodiment of the invention, the enzyme is a specific and/or selective peptidase of target tumor cells, stromal cells of the environment of the target tumor, neoangiogenic endothelial cells for example of the environment of the target tumor cells, macrophages or monocytes more particularly of the environment of the target tumor cells. In one embodiment of the invention, when the target tissue is a tumor, the enzyme is at least one peptidase that can be selected from the group of : neprilysin (CALLA or CD10), thimet oligopeptidase (TOP), leukotriene A4 hydrolase, endothelin converting enzymes, ste24 protease, neurolysin, mitochondrial intermediate peptidase, interstitial collagenases, collagenases, stromelysins, macrophage elastase, matrilysin, gelatinases, meprins, procollagen C-endopeptidases, procollagen N-endopeptidases, ADAMs and ADAMTs metalloproteinases, myelin associated metalloproteinases, enamelysin, tumor necrosis factor α-converting enzyme, insulysin, nardilysin, mitochondrial processing peptidase, magnolysin, dactylysin-like metalloproteases, neutrophil collagenase, matrix metallopeptidases, membrane-type matrix metalloproteinases, SP2 endopeptidase, prostate specific antigen (PSA), plasmin, urokinase, human fibroblast activation protein (FAPα), trypsin, chymotrypsins, caldecrin, pancreatic elastases, pancreatic endopeptidase, enteropeptidase, leukocyte elastase, myeloblastin, chymases, tryptase, granzyme, stratum corneum chymotryptic enzyme, acrosin, kallikreins, complement components and factors, alternative-complement pathway c3/c5 convertase, mannose-binding protein-associated serine protease, coagulation factors, thrombin, protein c, u and t-type plasminogen activator, cathepsin G, hepsin, prostasin, hepatocyte growth factor-activating endopeptidase, subtilisin/kexin type proprotein convertases, furin, proprotein convertases, prolyl peptidases,

acylaminoacyl peptidase, peptidyl-glycaminase, signal peptidase, n-terminal nucleophile aminohydrolases, 20s proteasome, γ-glutamyl transpeptidase, mitochondrial endopeptidase, mitochondrial endopeptidase la, htra2 peptidase, matriptase, site 1 protease, legumain, cathepsins, cysteine cathepsins, calpains,_ubiquitin isopeptidase T, caspases, glycosylphosphatidylinositol:protein transamidase, cancer procoagulant, prohormone thiol protease, γ-Glutamyl hydrolase, bleomycin hydrolase, seprase, cathepsin D, pepsins, chymosyn, gastricsin, renin, yapsin and/or memapsins, these enzymes being well-known for one skilled in the art.

The term "oligopeptide" is used herein to refer to a polymer of amino acid residues. The term refers to polymers of naturally occurring amino acids as well as to polymers of non-naturally occurring amino acids or to polymers in which one or more amino acid residue is an artificial chemical mimetic of a corresponding naturally occurring amino acid.

The term "amino acid" refers to naturally occurring and non-natural amino acids, as well as amino acid analogs in L or D configuration and amino acid mimetics that function in a manner similar to amino acids, more specifically naturally occurring amino acids.

"Naturally occurring amino acids" are those amino acids encoded by the genetic code as well as those that are later modified, for example after synthesis, *e.g.* hydroxyproline, γ-carboxyglutamate, and *O*-phosphoserine, whereas non-natural amino acids are not encoded by the genetic code.

"Amino acid analogs" have the same basic chemical structure as naturally occurring amino acids, *i.e.*, hydrocarbon chain comprising an amino group and a carboxyl group, some of the carbons being substituted or not, *e.g.*, homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs have modified lateral chains (*e.g.*, norleucine) or modified backbones, but retain the same basic chemical structure as a naturally occurring amino acid (*e.g.* beta amino acids, amino acids in D conformation).

"Amino acid mimetics" have a structure that is different from the general chemical structure of an amino acid, but that functions in a manner similar to amino

acids, more specifically naturally occurring amino acids *e.g.* benzamidine arginine, azetidine 2-carboxylic acid, D-cyclohexylglycine.

The referenced amino acids are represented in this invention either in three-letter code or in one-letter code, these codes are well known to one skilled in the art.

The cleavable oligopeptide moiety of the compounds of the invention preferably comprises, or alternatively consists of, between 2 and 10 amino acids, and more preferably between 3 to 7 amino acids.

In some embodiments of the invention, the cleavable oligopeptide comprises, or alternatively consists of, one or more of the following amino acid sequences (preferably in conformation L): Arg-Leu, Arg-Phe, Arg-Val, Ala-Phe, Ala-Ala, Ala-Asn, Ala-Leu, Ala-Tyr, Asn-Leu, Cys-Arg, Cys-Asp, Cys-Phe, Gln-Phe, Gln-leu, Gly-Asp, Gly-Phe, Gly-Leu, Gly-Gln, Gly-Gly, Gly-Pro, His-Ser, Ile-Ala, Leu-Ala, Leu-Gln, Leu-Gly, Leu-Leu, Leu-Phe, Leu-Tyr, Leu-Lys, Lys-Leu, Met-Leu, Pro-Phe, Pro-Tyr, Pro-Leu, Phe-Leu, Phe-Phe, Tyr-Ile, Tyr-Pro, Tyr-Leu, Tyr-Gly, Val-Tyr, Val-Phe, Ser-Ser, Ser-Leu and Ser-Lys.

In a preferred embodiment of the invention, the oligopeptide comprises, or alternatively consists of, one or more of the following sequences (preferably in conformation L): (Leu)y-(Ala-Leu)x-Ala-Leu or (Leu)y-(Ala-Leu)x-Ala-Phe wherein Leu is leucine, Ala is alanine, Phe is phenylalanine, y = 0, 1, 2, 3, 4 or 5 and x = 1, 2, 3, 4 or 5.

Typically, the oligopeptide comprises, or alternatively consists of, one or more of the following sequences (preferably in conformation L): Ala-Phe-Lys, Ala-Leu-Ala-Leu, Ala-Leu-Lys-Leu-Leu, Ala-Tyr-Gly-Gly-Phe-Leu, His-Ser-Ser-Lys-Leu-Gln-Leu, Gly-Pro-Leu-Gly-Ile-Ala-Gly-Gln, Cys-Asp-Cys-Arg-Gly-Asp-Cys-Phe-Cys, Ala-Ala-Asn-Leu or Lys-Ala-Leu-Ala-Leu or conservative substitution variants thereof, more specifically, variants wherein each amino acid residue, independently, can be replaced by another residue with identical chemical properties. For example, the Ala residue can be substituted for by any other non-polar residue, the Lys residue can be substituted for by any other basic residue, Asp can be substituted for by another acidic residue and so on.

In another preferred embodiment of the invention, the oligopeptide comprises, or alternatively consists of, one or more of the following sequences (preferably in

conformation L): Ala-Phe-Lys, Ala-Leu-Ala-Leu (SEQ ID NO:1) or beta-Ala-Leu-Ala-Leu (SEQ ID NO:2), Ala-Leu-Lys-Leu-Leu (SEQ ID NO:3), Ala-Tyr-Gly-Gly-Phe-Leu (SEQ ID NO:4), His-Ser-Ser-Lys-Leu-Gln-Leu (SEQ ID NO:5), Gly-Pro-Leu-Gly-Ile-Ala-Gly-Gln (SEQ ID NO:6), Cys-Asp-Cys-Arg-Gly-Asp-Cys-Phe-Cys (SEQ ID NO:7) and Ala-Ala-Asn-Leu (SEQ ID NO:8), Lys-Ala-Leu-Ala-Leu (SEQ ID NO:9).

In a particular embodiment of the invention, compounds of formula (II) are those wherein X comprises, or consists of, the sequence of SEQ ID NO: 1: Ala-Leu-Ala-Leu. This amino acid sequence is cleaved in particular by neprilysin, thimet oligopeptidase or cathepsin B enzymes.

One skilled in the art knows other amino acid sequences that can be cleaved selectively by a specific enzyme of tumor cells, such as those described in WO 00/33888 [6] filed by COULTER PHARMACEUTICAL (see amino acid sequences described pages 13 to 15), WO 01/68145 [8] filed by DUPONT PHARMACEUTICALS (see amino acid sequences described SEQ ID Nos 1 to 210), WO 01/95943 [9] filed by CORIXA (see amino acid sequences described pages 16 to 18), WO 02/00263 [10] filed by CORIXA (see amino acid sequences described table 1, page 16), WO 02/100353 [11] filed by MEDAREX (see amino acid sequences described SEQ ID Nos 1 to 36), WO 02/07770 [12] filed by PHARMACIA & UPJOHN (see amino acid sequences described page 3), WO 99/28345 [13] filed by MERCK & CO (see amino acid sequences described SEQ ID Nos 1 to 125), or WO 03/094972 [14] filed by BOEHRINGER INGELHEIM PHARMA, each incorporated herein by reference in their entirety and for all purposes.

Furthermore, one skilled in the art can refer to Handbook of Proteolytic Enzymes, (A.J. Barrett, N.D. Rawlings, and J.F. Woessner eds. Academic Press, 1998; A.J. Barrett, N.D. Rawlings, and J.F. Woessner, Second Edition eds. Elsevier, 2004) [15] incorporated herein by reference in its entirety and for all purposes) to characterize the substrate-specificity of enzymes.

For the compounds of the invention to be effective, they typically undergo *in vivo* modification releasing the active therapeutic agent or an active derivative of the therapeutic agent which is able to exert its biological activity on the target cell either after entry into the target cell or without entry (*e.g.*, if the therapeutic agent is a ligand of a

membrane receptor like tumor necrosis factors). In one embodiment of the invention, an initial cleavage within the cleavable oligopeptide portion of the compound of the invention may result in a fragment of the compound that is competent for transport into the target cell. Alternatively, further cleavage by one or more peptidases may be required to result in a transport-competent fragment of the compound. The active or transport-competent fragment of the compound comprises at least the therapeutic agent and a part of the compound of formula (II) that can enter into or interact with the target cells to exert a therapeutic effect directly or upon further conversion within the target cell.

The target cells-associated enzymes defined above are able to selectively cleave the cleavable oligopeptide moiety so as to make possible the release of the therapeutic agent (or marker) or the release of the therapeutic agent (or marker) linked to a portion of the cleavable oligopeptide (and/or to the linker moiety L2, if any). The expression "release of the therapeutic agent (or marker) linked to a portion of the cleavable oligopeptide" is explained by the following example. If the cleavable oligopeptide moiety is Ala-Leu-Ala-Leu, the therapeutic agent is doxorubicin (whereby the therapeutic agent directly linked to the cleavable oligopeptide is Ala-Leu-Ala-Leu-doxorubicin) and the enzyme is CD10 (neprilysin), then this enzyme cleaves the sequence of amino acids between Ala-Leu-Ala and Leu, thus releasing a Leu-doxorubicin product (*i.e.*, the therapeutic agent linked to a part of the cleavable oligopeptide, the last Leu in the example).

The compounds of the invention comprise a therapeutic agent or marker moiety. "Therapeutic agent" is intended to mean a compound that, when present in a therapeutically effective amount, produces a desired therapeutic effect on a mammal, and whose action site is located or whose effect will be exerted on the surface of, or inside, target cells. By way of example, a therapeutic agent can be selected from the following group: a chemical agent, a polypeptide, a protein, an antibody, a nucleic acid, an antibiotic or a virus.

Said therapeutic agent is preferably an agent with anti-tumor, anti-angiogenic or anti-inflammatory therapeutic activity. The agent can have an extracellular target (for example a receptor) or intracellular action site. For example, a therapeutic agent according to the invention may be selected from vinca alkaloids such as vincristine,

vinblastine, vindesine, vinorelbine; taxanes or taxoids such as paclitaxel, docetaxel, 10-deacetyltaxol, 7-*epi*-taxol, baccatin III, 7-xylosyltaxol, isotaxel; alkylating agents such as ifosfamide, melphalan, chloroaminophene, procarbazine, chlorambucil, thiophosphoramide, busulfan, dacarbazine (DTIC), mitomycins including mitomycin C, nitroso-ureas and derivatives thereof (for example, estramustine, BCNU, CCNU, fotemustine, streptonigrin); platinum derivatives such as cisplatin and the like (for example, carboplatin, oxaliplatin); antimetabolites such as methotrexate, aminopterin, 5-fluorouracil, 6-mercaptopurine, raltitrexed, cytosine arabinoside (or cytarabine), adenosine arabinoside, gemcitabine, cladribine, pentostatin, fludarabine phosphate, hydroxyureas; inhibitors of topoisomerase I or II such as the camptothecin derivatives (for example, irinotecan and topotecan or 9-dimethylaminomethyl-hydroxy-camptothecin hydrochloride), epipodophyllotoxins (for example etoposide, teniposide), amsacrine; mitoxantrone; L-canavanine; antibiotic agents such as anthracyclines and, for example, doxorubicin, THP-adriamycin, daunorubicin, idarubicin, rubidazone, pirarubicin, zorubicin and aclarubicin, the analogs of anthracyclines and, for example, epiadriamycin (4'epi-adriamycin or epirubicin) and mitoxantrone, bleomycins, actinomycins including actinomycin D, streptozotocin, calicheamycin, duocarmycins, combretastatin; L-asparaginase; hormones; pure inhibitors of aromatase; androgens, analog-antagonists of LH-RH; cytokines such as interferon alpha (IFN-alpha), interferon gamma (IFN-gamma), interleukin 1 (IL-1), IL-2, IL-4, IL-6, IL-10, IL-12, IL-15, tumor necrosis factor-alpha (TNF-alpha), IGF-1 antagonists (insulin-like growth factor); proteasome inhibitors; farnesyl-transferase inhibitors (FTI); epothilones; maytansinoids; discodermolide; fostriecin; inhibitors of tyrosine kinases such as STI 571 (imatinib mesylate); receptor tyrosine kinase inhibitors such as lapatinib, erlotinib, sorafenib, vandetanib, canertinib, PKI166, gefitinib, sunitinib, EKB-569; Bcr-Abl kinase inhibitors such as dasatinib, nilotinib, imatinib; aurora kinase inhibitors such as VX-680, CYC116, PHA-739358, SU-6668, JNJ-7706621, MLN8054, AZD-1152, PHA-680632; CDK inhibitors such as flavopirodol, seliciclib, E7070, BMS-387032; MEK inhibitors such as PD184352, U-0126; mTOR inhibitors such as CCI-779 or AP23573; Kinesin spindle inhibitors such as SB 743921, MK-0731 or ispinesib; RAF/MEK inhibitors such as; Sorafenib, CHIR-265, PLX-4032, CI-1040, PD0325901 or ARRY-142886; bryostatin; L-779450; LY333531; velcade; endostatins; proteins; peptides; antibodies; anti-

inflammatory cytokines; the spindle poison colchicine; the HSP 90 binding agent geldanamycin, macrocyclic polyethers such as halichondrin B, eribulin.

Particularly preferred therapeutic agents are doxorubicin, mitomycin C, ispinesib, geldanamycin, colchicine, vinblastine, isotaxel, paclitaxel, docetaxel and derivatives thereof.

"Marker" is intended to mean a compound useful in the characterization of tumors or other medical conditions, for example, diagnosis, progression of a tumor, and assay of the factors secreted by tumor cells. A marker is further defined as enzymes, antibodies, fluorescent or phosphorescent chemical molecules, and molecules that can be used in scintigraphy. Examples of markers include, but are not limited to, coumarin, 7-amino-trifluoromethyl coumarin, paranitroanilide, 8-naphthylamide and 4-methoxy naphthylamide, fluoresceine, biotin, rhodamine, tetramethylrhodamine, GFP (green fluorescent protein), agents that are used in scintigraphy such as radioactive isotopes, and the derivatives of these compounds. Such markers can be used as part of a diagnostic kit.

When the cleavable oligopeptide moiety of a compound of the invention is directly linked to the therapeutic agent or marker, i.e. when L2 in formula (II) is a covalent bond, said covalent bond between said oligopeptide moiety and said therapeutic agent or marker can then be produced at the N-terminal or C-terminal end of the cleavable oligopeptide, according to its orientation, or at any other site of the oligopeptide (for example at the lateral chain of one of the amino acids).

A direct chemical reaction between a therapeutic agent and an oligopeptide is possible when each possesses a functional group capable of reacting with the other. For example, a nucleophilic group, such as an amino or sulfhydryl group, can be reacted with a carbonyl-containing group, such as an anhydride or an acid halide, or with an alkyl group containing a good leaving group (*e.g.*, a halide). Examples of bonds between an oligopeptide and a therapeutic agent may be found in PCT Publication No WO 96/05863 [3], which is incorporated herein by reference. Preferred bonds are amide, ester or thioester, carbonate, carbamate, hydrazone, oxime, ether and thioether or disulfide.

The compounds of the invention present a TA derived moiety directly or indirectly linked to an oligopeptide moiety, said oligopeptide moiety being directly or

indirectly linked to a therapeutic agent or marker. A direct link between two parts of the molecule means that one of these parts comprises a reactive group which can allow formation of a covalent bond with a reactive group on the other part.

The terms "indirect linkage" or "indirectly linked" mean that two portions of the compound of the invention are covalently linked through a linking moiety. If the TA derived moiety and the cleavable oligopeptide are indirectly linked, then a linking moiety L_1 is present. In the same way, if the cleavable oligopeptide moiety and the therapeutic agent or marker are indirectly linked, then a linking moiety L_2 is present. According to one embodiment, the compounds of the invention have the TA derived moiety and the cleavable oligopeptide moiety indirectly linked. In another embodiment, the compounds of the invention have indirect linkage of the cleavable oligopeptide to the therapeutic agent or marker. In yet another embodiment, the compounds of the invention have indirect linkage of the TA derived moiety and the cleavable oligopeptide and indirect linkage of the cleavable oligopeptide to the therapeutic agent or marker.

In a particular embodiment of the invention, the orientation of the cleavable oligopeptide may be reversed so that the TA derived moiety is indirectly linked to the cleavable oligopeptide at the C-terminus of the cleavable oligopeptide and the therapeutic agent or marker is directly or indirectly linked to the N-terminus of the cleavable oligopeptide. In a particular embodiment of the invention, a first attachment site of the cleavable oligopeptide may be the C-terminus of the cleavable oligopeptide and a second attachment site of the cleavable oligopeptide may be the N-terminus of the cleavable oligopeptide.

According to the present invention, L_1 and L_2 are independently a covalent bond or a linking moiety, said linking moiety should include at least two organic functional groups, one organic functional group that can bind to the 1,2,3,4-cyclobutanetetracarboxylic acid and a second organic functional group that allows binding to the oligopeptide moiety (L1) or one functional group that can bind to the oligopeptide moiety and a second organic functional group that allows binding to D (L2).

The term "linking moiety" includes di- and multivalent organic radicals independently selected from substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl and substituted or unsubstituted aryl, aldehydes, acids, esters and anhydrides, ketone, acyl halide, quinone, alkylene, cycloalkyl, heterocycloalkyl, thiazolidine, oxazolidine, imidazole, sulphydryl or carboxyl groups, such as maleimido acid derivatives, and succinimido derivatives or may be derived from succinimidyl esters and the like. The L2 linking moiety should include at least two organic functional groups that can bind the peptide moiety to the drug. One organic functional group, which allows binding to the peptide moiety, may preferably be selected from, but is not limited to, the group comprising an amine or hydroxyl or thiol group. The other organic functional group, which allows binding to the drug, may preferably be selected from, but is not limited to, the group comprising an amine, hydroxyls, thiols, carboxyl group, carbonyl group, halide, maleimide. These two functional groups are attached to a backbone which may comprise from 0 to 50 carbons and heteroatoms (N, O, S), arranged in a straight or branched chain or cyclic moiety or any combination thereof, saturated or unsaturated. According to one embodiment of the invention, L1 and L2 each contain two organic functional groups the first organic functional group selected from an amine, hydroxyl or thiol group and the second organic functional group selected from an amine, hydroxyl, thiol, carboxyl, carbonyl, halide or maleimide group. These two functional groups are attached to a backbone which may comprise from 0 to 50 carbons and heteroatoms (N, O, S), arranged in a straight or branched chain or cyclic moiety or any combination thereof, saturated or unsaturated.

For example, the linking moiety may be selected from the group comprising diamino-alkyl, diamino-alkene, diamino-aryl, diamino-cycloalkyl, diamino-poly(ethyleneoxy), aminoalkanoic acid, aminoaryl-carboxylic acid, aminocycloalkyl-carboxylic acid, amino-poly(ethyleneoxy)-carboxylic acid, hydroxylamino-alkyl, hydroxylamino-alkene, hydroxylamino-aryl, hydroxylamino-cycloalkyl, hydroxyl-poly(ethyleneoxy)amine, thioamino-alkyl, thioamino-aryl, thioamino-cycloalkyl, alkanoyl halide, aminoalkyl halide, maleimidoalkanoic acid, maleimidocycloalkyl-carboxylic acid and maleimidoamino alkyl. Particularly, the linking moiety can also include aminobenzyl spacer, elongated electronic cascade and cyclisation spacer systems, cyclisation

elimination spacers, such as ω-amino aminocarbonyls, and *p*-aminobenzyoxycarbonyl linkers (de Groot *et al.*, J. Med. Chem. 43, 3093 (2000)) [16].

The linking moiety may comprise a short amino acid sequence from 1 to 10 amino acid residues that is not cleaved by the enzyme cleaving the cleavable oligopeptide moiety of a compound of formula (II). Accordingly the linking moiety comprises identical or different amino acids selected from natural amino acids in conformation L or D, non genetically encoded amino acids or amino acids that cannot be recognized by enzymes (*e.g.* peptidases) that are present in the circulatory structure, such as beta- or gamma-amino acids or the like. The term "natural amino acids in conformation D" denotes the amino acids that are normally coded by the genetic code but that instead of being naturally in conformation L are in conformation D. Generally, the non genetically encoded amino acids can be prepared by synthesis or can be derived from a natural source. Preferred among the natural amino acids in conformation D are the hydrophilic amino acids that are selected from among: D-serine, D-threonine, D-glutamine, D-asparagine, D-aspartic acid, D-glutamic acid, D-lysine, D-arginine and D-histidine. Particularly preferred amino acids in conformation D are D-serine and D-threonine. By way of example, the linking moiety may comprise a stretch of identical amino acids that are selected from among: (L-serine)x, (D-serine)x, or (L-threonine)x and (D-threonine)x, where x is an integer between 1 and 10, preferably x = 2 or 4.

Linking moieties have also been disclosed in de Groot *et al.*, J. Med. Chem. 42, 5277 (1999) [17]; de Groot *et al.*, J. Med. Chem. 43, 3093 (2000) [16] ; de Groot *et al.*, J. Org. Chem. 66, 8815, (2001) [18] ; PCT Publication No WO 02/083180 [19] ; Carl *et al.*, J. Med. Chem. Lett. 24, 479 (1981)[20] ; Dubowchik *et al.*, Bioorg & Med. Chem. Lett. 8, 3347 (1998) [21].

It will be evident to those skilled in the art that a variety of bifunctional or polyfunctional reagents, both homo- and hetero-functional can be employed as a linking moiety, such as those described in the catalog of the Pierce Chemical Co., Rockford, III., 2008 [22].

Preferably, the linking moiety according to this invention consists of, or alternatively comprises, at least one moiety selected from: sequences of amino acids; peptidomimetic agents; pseudopeptides; peptoids; substituted alkyl, aryl alkylene or

arylalkyl chains; polyalkyl glycols; polysaccharides; polyamino; polyols; polycarboxylates; and poly(hydroxy)esters.

The linking may space the 1,2,3,4-cyclobutanetetracarboxylic acid derived moiety or the therapeutic agent or marker from the oligopeptide in order to avoid interference in the interaction of the oligopeptide with enzymes associated with target cells. A linking moiety can also serve to allow or increase the coupling efficiency between two portions of the compounds of the invention. For example, if the therapeutic agent or marker (D in formula II) does not comprise a group allowing its direct binding to the cleavable peptide moiety (X in formula II), a linker moiety L2 comprising reactive groups compatible with both X and D moieties may be inserted for coupling these two parts.

In a preferred embodiment, the therapeutic agent or marker is directly linked to the cleavable oligopeptide moiety. It will be evident for one skilled in the art that this embodiment requires that the therapeutic agent or marker and the cleavable oligopeptide moiety present radicals which can allow formation of a covalent bond.

The term "alkyl" by itself or as part of another substituent, means, unless otherwise stated, a straight or branched chain, or cyclic hydrocarbon radical, optionally interrupted by at least one heteroatom including O, N, Si and S (as defined below), or combination thereof, which may be fully saturated, mono-or polyunsaturated and can include di- and multivalent radicals, having the number of carbon atoms designated (*i.e.* C_1-C_{10} means one to ten carbons). Examples of saturated hydrocarbon radicals of formula C_nH_{2n+1} include, but are not limited to, groups such as methyl, ethyl, n-propyl, isopropyl, n-butyl, t-butyl, isobutyl, sec-butyl, cyclohexyl, (cyclohexyl) methyl, cyclopropylmethyl, homologs and isomers of, for example, n-pentyl, n-hexyl, n-heptyl, n-octyl, and the like. An unsaturated alkyl group is one having one or more double bonds (*e.g.*, alkenyl groups of formula C_nH_{2n}) or triple bonds (*e.g.*, alkynyl groups of formula C_nH_{2n-2} wherein n is an integer from 1 to 50. Examples of unsaturated alkyl groups include, but are not limited to, vinyl, 2-propenyl, crotyl, 2-isopentenyl, 2-butadienyl, 2,4-pentadienyl, 3(1,4-pentadienyl), ethynyl, 1-and 3-propynyl, 3-butynyl, and the higher homologs and isomers. The term "alkyl" unless otherwise noted, is also meant to include those derivatives of alkyl defined in more detail below, such as "heteroalkyl". Alkyl groups, which are limited to hydrocarbon groups are termed "homoalkyl".

The term "alkyl" by itself or as part of another substituent includes mono- or divalent radical derived from an alkane. Among the divalent radical, one can cite as non limiting examples $-CH_2-$, $-CH_2CH_2-$, $-CH_2CH_2CH_2-$, $-CH_2CH_2CH_2CH_2-$.

Typically, an alkyl group will have from 1 to 24 carbon atoms, with those groups having 10 or fewer carbon atoms being preferred in the present invention. A "lower alkyl" is a shorter chain alkyl group, generally having eight or fewer carbon atoms.

The term "alkenyl" by itself or as part of another substituent means a divalent radical derived from an alkane, as exemplified, but not limited, by $-CH_2=CH_2-$, and further includes those groups described below as "heteroalkylene". Typically, an alkylene group will have from 2 to 24 carbon atoms, with those groups having 10 or fewer carbon atoms being preferred in the present invention. A "lower alkylene" is a shorter alkylene group, generally having eight or fewer carbon atoms.

The term "heteroalkyl" by itself or in combination with another term, means, unless otherwise stated, a stable straight or branched chain, or cyclic hydrocarbon radical, or combinations thereof, consisting of the stated number of carbon atoms and at least one heteroatom selected from the group consisting of O, N, Si and S, and wherein the nitrogen, carbon and sulfur atoms may optionally be oxidized and the nitrogen heteroatom may optionally be quaternized. The heteroatom(s) O, N and S and Si may be placed at any interior position of the heteroalkyl group or at the position at which the alkyl group is attached to the remainder of the molecule. Examples include, but are not limited to, $-CH_2-O-CH_3$, ethyleneglycol and the like. Up to two heteroatoms may be consecutive, such as, for example, $-CH_2-NH-OCH_3$ and $-CH_2-O-S(CH_3)_3$.

The term "heteroalkyl" may encompass poly(ethylene glycol) and its derivatives. Still further, for divalent alkyl and heteroalkyl groups, no orientation of the linking group is implied by the direction in which the formula of the group is written. For example, the formula $-C(O)_2R'-$ represents both $-C(O)_2R'-$ and $-R'C(O)_2-$.

The term "lower" in combination with the terms "heteroalkyl" refers to a moiety having from 1 to 8 carbon atoms.

The terms "alkoxy", "alkylamino" and "alkylthio" (or "thioalkoxy") are used in their conventional sense, and refer to those alkyl groups attached to the remainder of the molecule via an oxygen atom, an amino group, or a sulfur atom, respectively.

In general, an "acyl substituent" is also selected from the group set forth above. As used herein, the term "acyl substituent" refers to groups attached to, and fulfilling the valence of a carbonyl carbon that is either directly or indirectly attached to the compounds of the present invention.

The terms "cycloalkyl" and "heterocycloalkyl", by themselves or in combination with other terms, represent, unless otherwise stated, cyclic versions of substituted or unsubstituted "alkyl" and substituted or unsubstituted "heteroalkyl", respectively. Additionally, for heterocycloalkyl, a heteroatom can occupy the position at which the heterocycle is attached to the remainder of the molecule. Examples of cycloalkyl include, but are not limited to, cyclopentyl, cyclohexyl, 1-cyclohexenyl, 3-cyclohexenyl, cycloheptyl, and the like. Examples of heterocycloalkyl include but are not limited to, 1-piperidinyl, 3-piperidinyl, tetrahydrofuran-2-yl and the like. The heteroatoms and carbon atoms of the cyclic structures are optionally oxidized.

The terms "halo" or "halogen" by themselves or as part of another substituent, mean, unless otherwise stated, a fluorine, chlorine, bromine, or iodine atom. Additionally, terms such as "haloalkyl" are meant to include monohaloalkyl and polyhaloalkyl. For example, the term "halo(C_1-C_4)alkyl" is meant to include, but is not limited to, trifluoromethyl, 2,2,2-trifluoroethyl, 4-chlorobutyl, 3-bromopropyl, and the like.

The term "aryl" means, unless otherwise stated, a substituted or unsubstituted polyunsaturated, aromatic, hydrocarbon substituent which can comprise a single ring or multiple rings (preferably from 1 to 3 rings) which are fused together or linked covalently. The term "heteroaryl" refers to aryl groups (or rings) that contain from one to four heteroatoms selected from N, O, and S, wherein the nitrogen, carbon and sulfur atoms are optionally oxidized, and the nitrogen atom(s) is optionally quaternized. A heteroaryl group can be attached to the remainder of the molecule through a heteroatom. Non-limiting examples of aryl and heteroaryl groups include phenyl, 1-naphthyl, 4-biphenyl, 1-pyrrolyl, 2-imidazolyl, pyrazinyl, 2-oxazolyl, 2-pyridyl, 4-pyrimidyl, 5-benzothiazolyl, and the like.

Substituents for each of the above noted aryl and heteroaryl ring systems are selected from the group of acceptable substituents described below. "Aryl" and "heteroaryl" also encompass ring systems in which one or more non-aromatic ring systems are fused, or otherwise bound, to an aryl or heteroaryl system.

For brevity, the term "aryl" when used in combination with other terms (*e.g.*, aryloxy, arylthioxy, arylalkyl) includes both aryl and heteroaryl rings as defined above.

Thus, the term "arylalkyl" is meant to include those radicals in which an aryl group is attached to an alkyl group (*e.g.*, benzyl, phenethyl, pyridylmethyl and the like) including those alkyl groups in which a carbon atom (*e.g.*, a methylene group) has been replaced by, for example, an oxygen atom (*e.g.*, phenoxymethyl, 2-pyridyloxymethyl, 3-(1-naphthyloxy)propyl, and the like).

Each of the above radicals (*e.g.*, "alkyl", "heteroalkyl", "aryl" and "heteroaryl") include both substituted and unsubstituted forms of the indicated radical. Preferred substituents for each type of radical are provided below. Substituents are independently the same or different and are preferably alkyl group, alkylene group, heteroalkyl group, alkoxy group, alkylamino group, alkylthio group, acyl group, cycloalkyl and heterocycloalkyl group, aryl, heteroaryl group, halogen.

The functional groups on the linking moiety used to form covalent bonds may correspond to different types of functional groups. For example, it will be evident to those skilled in the art that coupling can be effected through functional groups such as amino, carboxyl, carbonyl, hydrazine, hydroxylamino, quinone, hydroxyl, thiol, halide, imine, isocyanate, nitrile, phosphate, sulfonic, sulfhydryl, maleimido groups. Functional groups have also been disclosed in March's Advanced Organic Chemistry: Reactions, Mechanisms and Structure (6^{th} edition by Michael B. Smith and Jerry March, edition Wiley) [23].

By way of example, the TA derived moiety can be linked to a suitable cleavable oligopeptide moiety either directly or indirectly via a linking moiety L_1. If present, the linking moiety L_1 according to the present invention covalently links the TA derived moiety to the cleavable oligopeptide. It is preferably hydrophilic and stable in the circulatory structure (*e.g.* the blood circulation or bloodstream). A linking moiety is "stable in the circulatory structure" when less than about 20%, preferably less than about 10%, preferably even less than about 2% (by moles), of the linking moiety is degraded or cleaved (in particular by enzymes) in the circulating blood of a mammal or during its

preservation in human blood at about 37°C, for more than about 2 hours. More preferably the linker is not degraded or cleaved (in particular by enzymes) in the circulating blood or during its preservation in human blood at about 37°C, for more than about 2 hours. In general, the cleavable oligopeptide moiety can be conjugated to the TA derived moiety, for example, by any suitable technique, with appropriate consideration of the need for pharmacokinetic stability and reduced overall toxicity to the patient.

By way of example, a therapeutic agent or marker can be linked (*i.e.* conjugated or coupled) to the cleavable oligopeptide moiety of the invention by any suitable technique known from one skilled in the art, with appropriate consideration of the need for pharmacokinetic stability and reduced overall toxicity to the patient. A therapeutic agent or marker can be linked to the oligopeptide either directly or indirectly via a linking moiety L_2.

The linking moiety L_2 may have several functions such as to facilitate the cleavage between the oligopeptide and the therapeutic agent or marker, to provide a suitable chemical bonding means between the oligopeptide and the therapeutic agent or marker, to improve the synthesis process of the compound of the invention, to improve the physical properties of the substance of the therapeutic agent or marker, or to provide an additional mechanism for the intracellular or extracellular release of the therapeutic agent or marker at or near the target cells. Advantageously it can be desirable to use a linking moiety L_2 which is cleavable during or upon penetration into a cell of the therapeutic agent linked to a portion of the cleavable oligopeptide, or which is gradually cleavable over time in the extracellular environment. The mechanisms for the intracellular release of a therapeutic agent from these linking moieties comprise cleavage by reduction of a disulfide bond, by irradiation of a photolabile bond, by hydrolysis of derivatized amino acid side chains and acid-catalyzed hydrolysis.

More preferred linking moieties L_2 are *N,N'*-ethylenediamine, ethanolamine, aminocaproic acid, 4-aminobenzyl alcohol, 6-amino-1-hexanol, 1,3-diaminopropane, cysteamine, 1,4-diaminobutane, *N*-methyl-1,3-propanediamine, *N*-methylethylenediamine, 4-aminobenzylamine.

The invention encompasses also pharmaceutically acceptable basic or acidic addition salts, hydrates, solvates, or stereoisomers of said compounds of the invention.

The terms "pharmaceutically acceptable salts" includes salts of the compounds of the invention which are prepared with non toxic acids or bases, depending on the particular substituents found on the compounds described herein. When compounds of the present invention contain relatively acidic functionalities, base addition salts can be obtained by contacting the neutral form of such compounds with a sufficient amount of the desired base, either neat or in a suitable inert solvent. Examples of pharmaceutically acceptable base addition salts include sodium, potassium, calcium, ammonium, organic amino, or magnesium salt, or a similar salt. When compounds of the present invention contain relatively basic functionalities, acid addition salts can be obtained by contacting the neutral form of such compounds with a sufficient amount of the desired acid, either neat or in a suitable inert solvent. Examples of pharmaceutically acceptable acid addition salts include those derived from inorganic acids like hydrochloric, hydrobromic, nitric, carbonic, monohydrogencarbonic, phosphoric, monohydrogenphosphoric, dihydrogenphosphoric, sulfuric, monohydrogensulfuric, hydriodic, or phosphorous acids and the like, as well as the salts derived from relatively non toxic organic acids like acetic, propionic, isobutyric, maleic, malonic, benzoic, succinic, suberic, fumaric, lactic, mandelic, phthalic, benzenesulfonic, p-tolylsulfonic, citric, tartaric, methanesulfonic, and the like. Also included are salts of amino acids such as arginate and the like, and salts of organic acids like glucuronic or galactunoric acids and the like. Certain specific compounds of the present invention contain both basic and acidic functionalities that allow the compounds to be converted into either base or acid addition salts.

The present invention also relates to methods for preparing compounds of formula (II) as defined above.

The compounds of the invention may be prepared by any method known in the art, and in particular as described in the examples of the present application.

For example, the cleavable oligopeptide moiety can be prepared using conventional solution- or solid-phase peptide synthesis methods. This peptide can then be reacted directly with the therapeutic agent or marker moiety and then a TA derived

moiety can be reacted with the resulting product in order to form compounds of the invention.

The skilled person is capable of preparing a wide variety of compounds of formula (II) utilising a variety of linker moieties. As stated above, an appropriate group on the therapeutic agent or marker may be selected for attachment to the cleavable oligopeptide moiety and if desired a linker L2 joined to said therapeutic agent or marker or to said oligopeptide, or both, prior to their coupling. Alternatively, the therapeutic agent or marker may also be modified so as to allow conjugation.

Advantageously, the combination of the 1,2,3,4-cyclobutanetetracarboxylic acid derived moiety with a cleavable oligopeptide (i.e. with an extracellular activation process by enzymes associated with target cells) allows the therapeutic use of the compounds of the invention to improve the delivery of a therapeutic agent selectively within the target cell environment (*e.g.*, target tissue).

The compounds of the invention are administered to the patient, carried through the blood stream in a stable form, and are modified by at least one enzyme selective of the target cell when in the vicinity of said target cell. Since the enzyme activity is only minimally, or preferably not, present within the extracellular vicinity of normal cells, the cleavable oligopeptide moiety is generally poorly, or not, cleaved outside target tissues and the therapeutic agent or marker reach normal cells only minimally.

The compounds of the invention are preferably stable in the circulatory structure. "Stable in the circulatory structure" means that less than 60%, preferably less than about 20%, preferably less than 20%, is degraded and/or cleaved in the circulating blood of a mammal or during its preservation in human blood at about 37°C for 6 hours and about 2% (by mole), preferably less than 2% (by mole) of a compound of the invention is degraded or cleaved in the circulating blood of a mammal or during its preservation in human blood at about 37°C for more than about 2 hours. The term "circulatory structure" is defined as body fluids, more particularly blood. In a specific embodiment the "circulatory structure" comprises tissues of the circulatory system.

The compounds of the invention are non toxic for healthy cells, non-coagulating (*i.e.* overcomes pro-coagulating properties of the therapeutic agent linked to the cleavable oligopeptide) and hindering (*i.e.*, preventing the therapeutic agent from acting on a cell until it has been released from the compound).

Another aspect of the invention provides a composition that comprises, or alternatively consists of, as an active ingredient, at least one compound of the invention.

In still another aspect, the invention contemplates the use of such compositions for the formulation and the preparation of biological, pharmaceutical, cosmetic, agricultural, diagnostic or tracing products.

In one aspect, the invention provides a pharmaceutical composition that comprises, or alternatively consists of, at least one compound according to the present invention that can be combined with a pharmaceutically acceptable carrier (vehicle, vector, diluent or excipient). The terms "pharmaceutically acceptable", as they refer to carrier (vehicle, vector, diluent or excipient), mean that the materials may be administered to a subject without subsequent production of undesirable physiological effects to a degree that would prohibit administration of the composition.

The compounds of the invention are useful to treat a mammalian subject, preferably a human subject. A subject can be treated with a pharmaceutically effective amount of a compound according to the invention. The expression "pharmaceutically effective amount" or "effective amount" means an amount that can induce the biological or medical response of a tissue, system, animal or human as expected by the research worker or the physician in attendance.

The compositions defined above can also comprise, or be combined with, at least one other medicinal active ingredient or at least one adjuvant that is well known to one skilled in the art, such as for example vitamins or anti-oxidizing agents, to be used in conjunction with a compound according to the invention to improve and/or to extend the treatment. Indeed, the compositions of the invention are particularly useful in that they have a very low toxicity.

The pharmaceutical compositions according to the invention can be used *in vivo* for preventive or curative purposes of specific diseases or disorders. Non-limiting examples of diseases or disorders for which the pharmaceutical formulations according to the invention may be used include cancers, metastases, cellular apoptosis disorders, degenerative diseases, tissue ischemia, infectious diseases of a viral, bacterial or fungal nature, inflammation disorders, pathological neo-angiogenesis and metabolic disorders.

The term "cancer" refers to any of a number of diseases that are characterized by uncontrolled, abnormal proliferation of cells, the ability of affected cells to spread locally or through the bloodstream and lymphatic system to other parts of the body (*i.e.*, metastases), as well as any of a number of characteristic structural and/or molecular features. A "cancerous cell" or "cancer cell" is understood as a cell having specific structural properties that can lack differentiation and be capable of invasion and metastasis. Examples of cancers are lung (*i.e.* small cell lung, non-small cell lung, bronchic cancers), pancreas, ovarian, breast, prostate, liver, kidney, colon, head, brain, bone, stomach, bladder cancers and non-Hodgkin lymphoma (see DeVita, V. *et al.* (eds.), 2005, Cancer Principles and Practice of Oncology, 6th. Ed., Lippincott Williams & Wilkins, Philadelphia, PA [24] ; this reference is herein incorporated by reference in its entirety for all purposes).

A further aspect of the invention provides a method for the treatment of cellular proliferative and/or differentiative disorders, disorders associated with bone metabolism, immune disorders, hematopoietic disorders, cardiovascular disorders, liver disorders, kidney disorders, muscular disorders, neurological disorders, hematological disorders, inflammatory diseases, viral diseases, pain or metabolic disorders, preferably for treating cancers comprising administering, preferably parenterally and more preferably intravenously, to a patient in need of such treatment an effective amount of a composition of the invention.

"Treating" or "treatment" includes the administration of the compositions or compounds of the present invention to a patient who has a disease or disorder (*e.g.*, cancer or metastatic cancer), a symptom of disease or disorder or a predisposition toward

a disease or disorder, with the purpose to cure, heal, alleviate, relieve, alter, remedy, ameliorate, improve or affect the disease or disorder, the symptoms of the disease or disorder, or the predisposition toward disease. "Treating" or "treatment" of cancer or metastatic cancer using the methods of the present invention refers to the treatment or amelioration or prevention of a cancer, including any objective or subjective parameter such as abatement, remission, diminishing of symptoms or making the disease condition more tolerable to the patient, slowing in the rate of degeneration or decline, or making the final point of degeneration less debilitating. The treatment or amelioration of symptoms can be based on objective or subjective parameters, including the results of an examination by a physician. Accordingly, the term "treating" includes the administration of the compounds or agents of the present invention to prevent or delay, to alleviate, or to arrest or inhibit development of the symptoms or conditions associated with neoplastic disease. The term "therapeutic effect" refers to the stabilization, reduction, elimination, or prevention of the disease, symptoms of the disease, or side effects of the disease in the subject.

The compounds of the invention are generally useful for the treatment of many medical conditions including cancer, neoplastic diseases, tumors, inflammatory diseases, and infectious diseases. Examples of preferred diseases for treatment are breast cancer, colorectal cancer, liver cancer, lung cancer such as small cell lung or non-small cell lung and bronchic cancers, prostate cancer, ovarian cancer, brain cancer such as neuroblastoma or glioblastoma., pancreatic cancer, colon cancer, stomach cancer, head and neck cancers, bladder cancer, non-Hodgkin's lymphoma, lymphoma, melanoma, leukaemia.

For example, several specific tumor types have been identified as being positive for CD10. Treatment for these types of tumors is especially advantageous with the compounds disclosed herein. For example, treatment for one of the following tumor types may be effected: B-cell lymphoblastic leukemia, T-cell lymphoblastic leukemia, lymphoma, including Hodgkin's lymphoma and non-Hodgkin's lymphoma, follicular lymphoma, Burkitt lymphoma, melanoma, ocular melanoma, cutaneous melanoma, colon adenocarcinomas, hepatocellular carcinomas, renal cell carcinoma, ovarian carcinoma,

prostate adenocarcinoma, liver carcinoma, transitional cell carcinoma, pancreatic adenocarcinoma, lung carcinoma, breast carcinoma and colon carcinoma.

In yet another aspect, the invention provides the use of at least one compound of the invention for the manufacture of a medicament for treating or preventing a number of disorders, including cellular proliferative and/or differentiative disorders, disorders associated with bone metabolism, immune disorders, hematopoietic disorders, cardiovascular disorders, liver disorders, kidney disorders, muscular disorders, neurological disorders, hematological disorders, inflammatory diseases, viral diseases, pain or metabolic disorders, preferably for treating cancers.

Furthermore, a particular embodiment of the invention relates to the use of at least one compound of formula II as defined above for the manufacture of a medicament for the treatment of cancers or infectious diseases.

Another particular embodiment of the invention relates to the use of a compound of formula II as defined above for the preparation of a medicament for treating breast cancers, colorectal cancers, liver cancers, lung cancers such as small cell or non-small cell, bronchic cancers, prostate cancers, ovarian cancers, brain cancers, and pancreatic cancers, colon cancers, head and neck cancers, stomach cancers, bladder cancers, non-Hodgkin's lymphomas, melanomas, leukaemias, neuroblastomas, or glioblastomas.

The administration of the compounds according to the invention can be done by any of the administration methods accepted for the therapeutic agents and generally known in the art. These processes include, but are not limited to, systemic administration, for example orally, nasally, parenterally or topical administration, for example by transdermal means or else by central administration, for example by an intracranial surgical path, or else by intraocular administration.

The parenteral administration is done generally by subcutaneous, intramuscular or intravenous injection, or by infusion. The injectable compositions can be prepared in standard forms, either in suspension or liquid solution or in solid form that is suitable for an extemporaneous dissolution in a liquid.

A possibility for parenteral administration uses the installation of a system with slow release or extended release that ensures the maintenance of a constant dose level.

Based on the administration method provided, the compounds of the invention can be in solid, semi-solid or liquid form.

For solid compositions, such as tablets, pills, powders or granules in the free state or included in capsules, the active ingredient can be combined with excipients, such as: a) diluents, for example lactose, dextrose, sucrose, mannitol, sorbitol, cellulose and/or glycine; b) lubricants, for example silica, talc, stearic acid, its magnesium or calcium salt and/or polyethylene glycol; c) binders, for example magnesium silicate and aluminum silicate, starch paste, gelatin, tragacanth gum, methyl cellulose, carboxymethyl cellulose that contains soda and/or polyvinyl pyrrolidone; if necessary, d) disintegrating agents, for example starch, agar, alginic acid or its sodium salt, or effervescent mixtures; and/or e) absorbents, coloring agents, aromatizing agents and sweetening agents.

For semi-solid compositions, such as suppositories, the excipient can be, for example, a fatty emulsion or suspension or can be based on polyalkylene glycol, such as polypropylene glycol.

The liquid compositions, in particular those intended for injection or to be included in a soft capsule, can be prepared by, for example, dissolution, dispersion, etc., of the compound according to the invention in a pharmaceutically pure solvent such as, for example, water, a solution of sodium chloride (NaCl), the physiological serum, aqueous dextrose, glycerol, ethanol, an oil and the like.

The compounds according to the invention can also be administered in the form of systems for release of the liposome or lipoplex type, such as in the form of small unilamellar vesicles, large unilamellar vesicles and multilamellar vesicles. The liposomes can be formed from a variety of phospholipids, containing in particular cholesterol, stearylamine or phosphatidylcholines.

The compositions according to the invention should be sterilized and/or can contain one or more non-toxic adjuvants and auxiliary substances such as agents for preservation, stabilization, wetting or emulsification; agents that promote dissolution; and salts to regulate osmotic pressure and/or buffers. In addition, they can also contain other substances that offer a therapeutic advantage. The compositions are prepared,

respectively, by standard processes of mixing, granulation or coating well known to those skilled in the art.

The dosage for the administration of compounds according to the invention is selected according to a variety of factors including the type, strain, age, weight, sex and medical condition of the subject; the severity of the condition to be treated; the method of administration; the condition of the renal and hepatic functions of the subject and the nature of the particular compound or salt that is used. A normally experienced physician or veterinarian will easily determine and prescribe the effective amount of the desired compound to prevent, disrupt or stop the progress of the medical condition that is to be treated.

A pharmaceutical composition as set forth above can contain from about 0.1 to about 100 % (by weight) of at least one compound of the invention.

By way of examples, when administered parenterally, the effective levels of the compounds according to the invention will be in the range of from about 0.002 mg to about 500 mg per kg of body weight and per day.

The compounds according to the invention can be administered in the form of single daily doses, or the total daily dosage can be administered in two, three, four or more doses per day.

In a further aspect, the invention provides a diagnostic agent comprising or alternatively consisting of at least one compound of formula II according to the present invention wherein D is a marker. Such a diagnostic agent can be used either *in vitro* or *in vivo*.

In yet another aspect, the invention provides a diagnostic kit that comprises the above diagnostic agent. More particularly, the diagnostic kit comprises, in one or more containers, a predetermined amount of a composition according to the invention.

Further advantages and characteristics of the invention will be apparent for one skilled in the art from the following examples, given by way of illustration and which are not intended to be limiting, and in which reference will be made to the accompanying drawings.

It will be clear that the invention may be practiced otherwise than as particularly described in the foregoing 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.

EXAMPLES

Materials

1,2,3,4-cyclobutanetetracarboxylic acid was purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France)

Peptides:

- Fmoc-Ala-Leu-Ala-Leu-OH (Fmoc-ALAL-OH): synthesis as described below
- Fmoc-β-Ala-Leu-Ala-Leu-OH (Fmoc-βALAL-OH): synthesis as for Fmoc-Ala-Leu-Ala-Leu-OH
- OMe-succinyl-β-Ala-Leu-Ala-Leu-OH (OMe-succinyl-βALAL-OH) was supplied by PeptySyntha (Brussels, Belgium)
- tBu-succinyl-β-Ala-Leu-Ala-Leu-OH (tBu-succinyl-βALAL-OH) was supplied by NeoMPS (Strasbourg, France)
- Fmoc-Ala-Ala-Asn-Leu-OH (Fmoc-AANL-OH): synthesis as described below
- Fmoc-Ala-Leu-Lys(ivDde)-Leu-Leu-OH (Fmoc-ALK(ivDde)LL-OH): synthesis as described below
- NH_2-Ala-Tyr-Gly-Gly-Phe-Leu-OH (NH_2-AYGGFL-OH) was supplied by UCB Bioproducts (Braine-l'Alleud, Belgium)
- Fmoc-Ala-Tyr-Gly-Gly-Phe-Leu-OH (Fmoc-AYGGFL-OH): synthesis as described below

- Fmoc-His-Ser-Ser-Lys(ivDde)-Leu-Gln-Leu-OH (Fmoc-HSSK(ivDde)LQL-OH): synthesis as described below

Drugs:

- mitomycin C (MMC): purchased from Kangbaoxiang Laboratories (Zhengzhou, China)
- paclitaxel: purchased from Hauser (Denver, CO, USA) and LC Laboratories (Woburn, MA, USA)
- doxorubicin (dox): purchased from Meiji Seika Kaisha Ltd. (Tokyo, Japan)
- ispinesib: synthesis as described in patent WO03070701 [25]
- geldanamycin: purchased from LC Laboratories (Woburn, MA, USA)
- vinblastine: purchased from Ajinomoto-Omnichem (Louvain-La-Neuve, Belgium)
- colchicine: purchased from Fluka (Saint-Quentin Fallavier, France)
- docetaxel: purchased from LC Laboratories (Woburn, MA, USA)
- isotaxel: synthesis as described in Hayashi Y. and al., J. Med. Chem. 46:3782-3784 (2003) [26]

Markers:

- 7-amino-4-methylcoumarin: purchased from Sigma (Saint-Quentin Fallavier, France)

Cell lines:

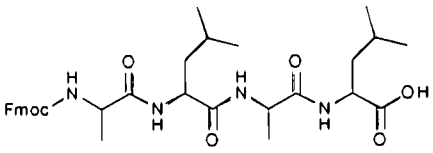
- HCT 116 human colon cancer cell line; ATCC Number: CCL-247
- LS 174T human colorectal carcinoma cell line; ATCC Number: CCl-188
- HT-29 human colorectal carcinoma cell line; ATCC Number HTB-38

I. Synthesis of peptide prodrug compounds

I.1 Method of synthesis of TA-peptide-doxorubicin

I.1.1 Synthesis of Fmoc-peptide-OH

I.1.1.1 Synthesis of Fmoc-ALAL-OH

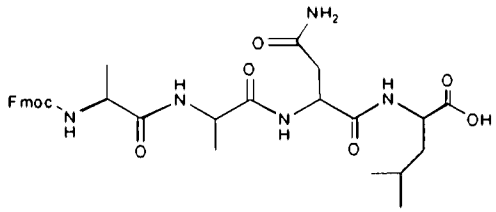


The Fmoc-Leu-Wang resin (5 g, 3.65 mmol, 1 eq) was swelled in dichloromethane (15 mL) for 5 minutes then in dimethylformamide (15 mL) for 5 minutes. The Fmoc group was removed by treatment with piperidine (4 mL) in dimethylformamide (16 mL) for 1 minute, the resin was then filtered, followed by the same treatment for 30 minutes. The resin was washed alternately with dimethylformamide (15 mL) and dichloromethane (15 mL) three times. Fmoc-Alanine-OH (3.408 g, 10.95 mmol, 3 eq) and 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (4.074 g, 9.85 mmol, 2.9 eq) were solubilised in dimethylformamide (15 mL) and added to the resin followed by *N,N*-diisopropylethylamine (3.8 mL, 21.9 mmol, 4 eq). The resin was then shaken for 30 minutes and washed alternately with dimethylformamide (15 mL) and dichloromethane (15 mL) three times. The solvent was removed and a second coupling was performed with Fmoc-Alanine-OH (2.272 g, 7.3 mmol, 2 eq), 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (2.718 g, 6.57 mmol, 1.9 eq) and *N,N*-diisopropylethylamine (2.5 mL, 14.6 mmol, 3 eq). The resin was then shaken for 30 minutes and washed alternately with dimethylformamide (15 mL) and dichloromethane (15 mL) three times. The same protocol was repeated with Fmoc-Leucine-OH then with Fmoc-Alanine-OH. After the last coupling, the resin was washed with dimethylformamide (15 mL), then with dichloromethane (15 mL) three times and dried. The peptide was cleaved from the resin with a solution of trifluoroacetic acid/triisopropylsilane/dichloromethane (95:1:4 v/v/v) (15 mL) two times during 30 minutes. The solvent was evaporated and the residue precipitated in *tert*-butyl methyl ether (100 mL). Dichloromethane was then added to the precipitate and the resulting mixture was co-evaporated several times to obtain a white solid which was used without further purification.

Purity: 90 % (determined by HPLC at 214 nm).

MS (ES^+): 610 $[MH]^+$

I.1.1.2 Synthesis of Fmoc-AANL-OH

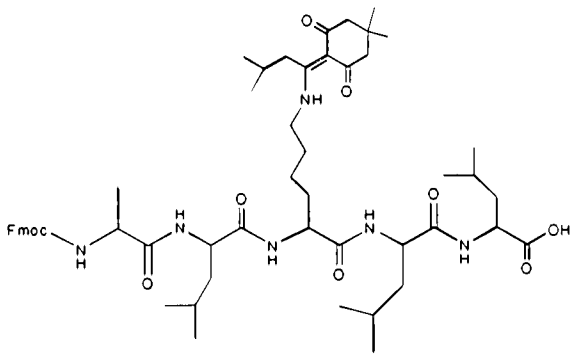


The Fmoc-Leu-Wang resin (1 g, 0.73 mmol, 1 eq) was swelled in dimethylformamide (20 mL) for 30 minutes. The Fmoc group was removed by treatment with piperidine (4 mL) in dimethylformamide (16 mL) for two times 3 minutes, and once for 7 minutes. The resin was washed three times with 20 mL dimethylformamide. Fmoc-Asparagine(Trt)-OH (0.87 g, 1.46 mmol, 2 eq) and 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (0.6 g, 1.46 mmol, 2 eq) were solubilized in dimethylformamide (20 ml) and *N.N*-diisopropylethylamine (0.5 mL, 2.92 mmol, 4 eq) was added to the amino acid. After 7 minutes, the mixture was added to the resin and shaken for 60 minutes. The resin was washed three times with dimethylformamide (20 mL). The Fmoc group was removed by treatment with piperidine (4 mL) in dimethylformamide (16 mL) The resin was washed three times with dimethylformamide (20 mL). The same protocol was repeated two times with Fmoc-Alanine-OH . After the last coupling, the resin was washed with dimethylformamide (15 mL), then with 15 mL dichloromethane (three times) and dried. The peptide was cleaved from the resin with a mixture of trifluoroacetic acid/triisopropylsilane/water (95:2.5:2.5 v/v/v) (20 mL) during 3 hours. The solvent was evaporated and the residue precipitated in water (100 mL). The product was filtered and dried.

Purity: 90 % (determined by HPLC at 214 nm).

MS (ES^+): 610 $[MH]^+$

I.1.1.3 Synthesis of Fmoc-ALK(ivDde)LL-OH

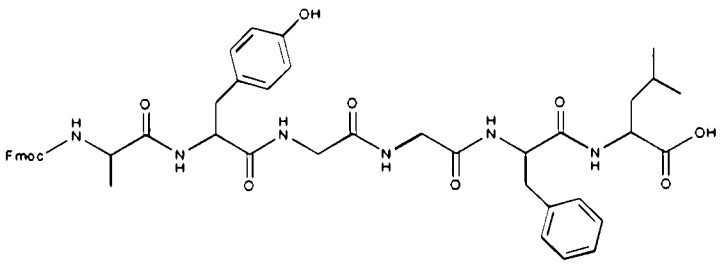


The Fmoc-Leu-Wang resin (1 g, 0.73 mmol, 1 eq) was swelled in dimethylformamide (20 mL) for 30 minutes. The Fmoc group was removed by treatment with piperidine (4 mL) in dimethylformamide (16 mL) for two times 3 minutes, and once for 7 minutes. The resin was washed three times with dimethylformamide (20 mL). Fmoc-Leucine-OH (0.52 g, 1.46 mmol, 2 eq) and 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (0.6 g, 1.46 mmol, 2 eq) were solubilized in dimethylformamide (20 mL) and *N,N*-diisopropylethylamine (0.5 mL, 2.92 mmol, 4 eq) added to the amino acid and after 7 minutes, the mixture was added to the resin and was shaken for 60 minutes. The resin was washed three times with dimethylformamide (20 mL). The Fmoc group was removed by treatment with piperidine (4 mL) in dimethylformamide (16 mL). The resin was washed three times with dimethylformamide (20 mL). The same protocol was repeated with Fmoc-Lysine(IvDde)-OH, Fmoc-Leucine-OH and Fmoc-Alanine-OH. After the last coupling, the resin was washed with dimethylformamide (15 mL), then dichloromethane (15 mL) three times and dried. The peptide was cleaved from the resin with a solution of trifluoroacetic acid/triisopropylsilane/water (95:2.5:2.5 v/v/v) (20 mL) during 3 hours. The solvent was evaporated and the residue precipitated in water (100 mL). The product was filtered and dried.

Purity: 90 % (determined by HPLC at 214 nm).

MS (ES^+): 986 $[MH]^+$

I.1.1.4 Synthesis of Fmoc-AYGGFL-OH

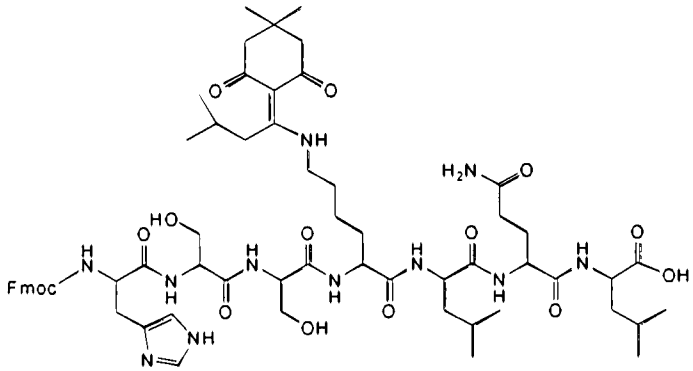


The peptide NH_2-AYGGFL-OH (1.5 g, 2.39 mmol, 1.1 eq) was solubilized in dimethylformamide (7 mL). *N*-(9-fluorenylmethylsuccinimide) (0.736 g, 2.178 mmol, 1 eq) and *N,N*-diisopropylethylamine (0.723 mL, 2.178 mmol, 2 eq) was added to the peptide. The solution was stirred for 1 hour at room temperature and directly precipitated in water (100 mL) and the solid filtered and dried.

Purity: 90 % (determined by HPLC at 214 nm).

MS (ES^+): 849$[MH]^+$

I.1.1.5 Synthesis of Fmoc-HSSK(ivDde)LQL-OH



The Fmoc-Leu wang resin (5 g, 3.65 mmol, 1 eq) was swelled in dichloromethane (15 mL) for 5 minutes then the solvent was removed. The same treatment was performed in dimethylformamide (15 mL) for 5 minutes. The Fmoc group was removed by treatment in piperidine (4 mL) and dimethylformamide (11 mL) for 1 minute. The solvent was removed and a second deprotection was performed with the same treatment for 30 minutes. The resin was washed alternately with dimethylformamide (15 mL) and dichloromethane (15 mL) three times. Fmoc-Glutamine-OH (4.04 g, 10.95 mmol, 3 eq) and 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (4.378 g, 10.57 mmol, 2.9 eq) were solubilised in dimethylformamide (15 mL) and added on the resin followed by *N,N*-diisopropylethylamine (3.8 mL, 21.9 mmol, 6 eq). The resin was shaken for 30 minutes and washed alternately with dimethylformamide (15 mL) and

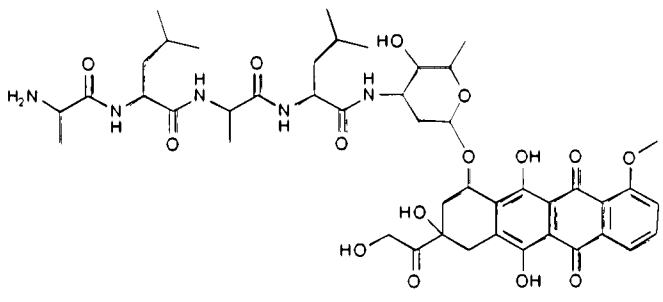
dichloromethane (15 mL) three times. The solvent was removed and a second coupling was performed with Fmoc-Glutamine-OH (2.693 g, 7.3 mmol, 2 eq), 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (2.718 g, 6.93 mmol, 1.9 eq) and *N,N*-diisopropylethylamine (2.5 mL, 14.6 mmol, 4 eq). The resin was shaken for 30 minutes and washed alternately with dimethylformamide (15 mL) and dichloromethane (15 mL) three times. The same protocol was repeated with Fmoc-Leucine-OH then with Fmoc-Lysine(ivDde)-OH, Fmoc-Serine-OH (twice) and Fmoc-Histidine-OH. After the last coupling, the resin was washed with dimethylformamide (3×15 mL), dichloromethane (3×15 mL) and dried. The peptide was cleaved from the resin with a solution of trifluoroacetic acid/triisopropylsilane/dichloromethane (95:1:4, 15 mL) twice during 30 minutes. The solvent was evaporated and the residue precipitated in *tert*-butyl methyl ether (100 mL). The filtrate was co-evaporated several times with dichloromethane to obtain a white solid which was used without further purification.

Purity: 93 % (determined by HPLC at 214 nm).

MS (ES^+): 1241 $[MH]^+$

I.1.2 Synthesis of peptide-doxorubicin conjugates

I.1.2.1 Synthesis of NH_2-ALAL-doxorubicin



Doxorubicin (0.479 g, 0.825 mmol, 1 eq) was solubilised in dimethylformamide (7 mL). A solution of Fmoc-ALAL-OH (0.600 g, 1 mmol, 1.2 eq) in dimethylformamide (1.4 mL) and H_2O (1.6 mL) was added to the doxorubicin solution followed by *N,N*-diisopropylethylamine (0.461 mL, 2.64 mmol, 3.2 eq). The solution was stirred at 4°C for 10 minutes. A solution of *O*-(7-Azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (0.376 g, 1 mmol, 1.2 eq) in dimethylformamide (1.6 mL) was added dropwise at 4°C. The solution was stirred for 40 minutes at room temperature and directly precipitated in *tert*-butyl methyl ether (100 mL) at 4°C. The resulting red oil was

evaporated. To the red oil was added: a solution of dimethylformamide (10 mL) and piperidine (4.2 mL, 41.25 mmol, 50 eq) and the solution was stirred for exactly 5 minutes at room temperature. The solution was directly quenched with HCl 6N (7 mL, 42 mmol, 50 eq) at 4°C (added slowly due to the exothermic nature of the reaction). The pH of the solution was adjusted to 6 with HCl 1N. The resulting red solution was precipitated in *tert*-butyl methyl ether (200 mL) at 4°C three times and the resulting red solid filtered and dried.

Purity: 95 % (determined by HPLC at 214 nm).

MS (ES^+): 913 $[MH]^+$

I.1.2.2 Synthesis of NH_2-AANL-doxorubicin

Prepared as described in paragraph I.1.2.1.

I.1.2.3 Synthesis of NH_2-ALK(ivDde)LL-doxorubicin

Prepared as described in paragraph I.1.2.1.

I.1.2.4 Synthesis of NH_2-AYGGFL-doxorubicin

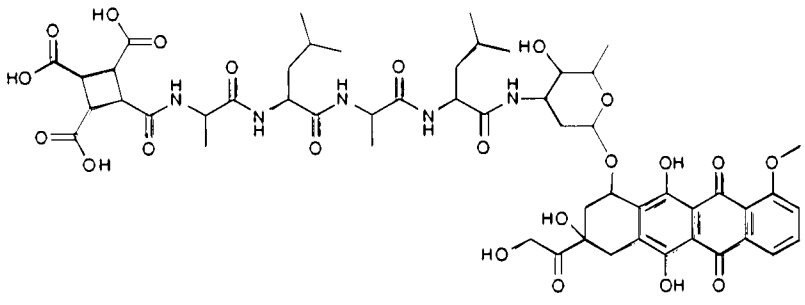
Prepared as described in paragraph I.1.2.1.

I.1.2.5 Synthesis of NH_2-HSSK(ivDde)LQL-doxorubicin

Prepared as described in paragraph I.1.2.1.

I.1.3 Synthesis of TA-peptide-doxorubicin conjugates

I.1.3.1 Synthesis of TA-ALAL-doxorubicin



NH_2-ALAL-doxorubicin (0.400 g, 0.44 mmol, 1 eq) was dissolved in dimethylformamide (10 mL). *N,N*-diisopropylethylamine (0.230 mL, 1.32 mmol, 3 eq) was added followed by 1,2,3,4-cyclobutanetetracarboxylic acid (1.021 g, 4.4 mmol, 10 eq). Then 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (0.237 g, 0.58

mmol, 1.3 eq) was added. The solution was stirred at room temperature for 30 minutes. Dichloromethane (30 mL) and water (15 mL) were added, the aqueous phase alkalinised to pH 8.5 with NaOH 1M and extracted four times with dichloromethane. The aqueous layer was purified by flash chromatography with silica C_{18} (H_2O/acetonitrile 90:10 to 60:10 v/v). The fractions were analyzed by HPLC and the pure fractions were pooled and lyophilized to generate a red solid.

Purity: 96 % (determined by HPLC at 214 nm).

MS (ES^+): 1127 $[MH]^+$

I.1.3.2 Synthesis of TA-AANL-doxorubicin

Prepared as described in paragraph I.1.3.1.

I.1.3.3 Method of synthesis of TA-ALKLL-doxorubicin

I.1.3.3.1 Synthesis of TA-ALK(ivDde)LL-doxorubicin

Prepared as described in paragraph I.1.3.1.

I.1.3.3.2 Synthesis of TA-ALKLL-doxorubicin

TA-ALK(ivDde)LL-doxorubicin (0.4 mmol, 1 eq) was solubilised in dimethylformamide (10 mL) and 350 μL hydrazine hydrate was added. The solution was stirred for 5 minutes at room temperature and was quenched with 50 mL of a 10% (v/v) lactate solution, pH 3. The reaction mixture was loaded on YMC®Gel ODS-A (C18 phase, 12 nm, s-50 µm) and the compound was eluted with methanol. The solvent was removed by rotary evaporation. TA-ALKLL-dox was collected in 30% DMF/water and pH adjusted to 7. The compound was purified by reverse-phase preparative HPLC (C18) and freeze-dried.

I.1.3.4 Synthesis of TA-AYGGFL-doxorubicin

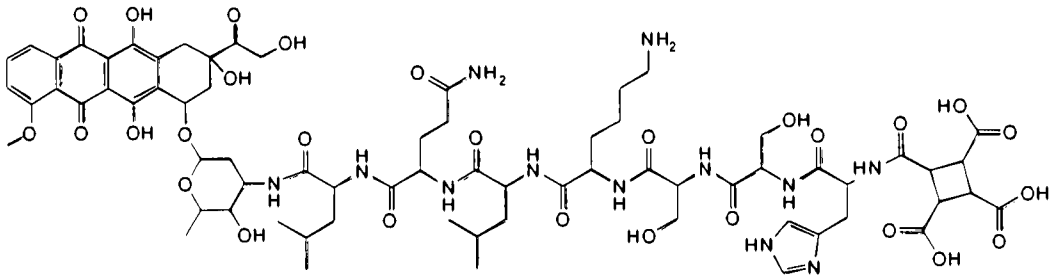
Prepared as described in paragraph I.1.3.1.

I.1.3.5 Method of synthesis of TA-HSSKLQL-doxorubicin

I.1.3.5.1 Synthesis of TA-HSSK(ivDde)LQL-doxorubicin

Prepared as described in paragraph I.1.3.1.

I.1.3.5.2 Synthesis of TA-HSSKLQL-doxorubicin

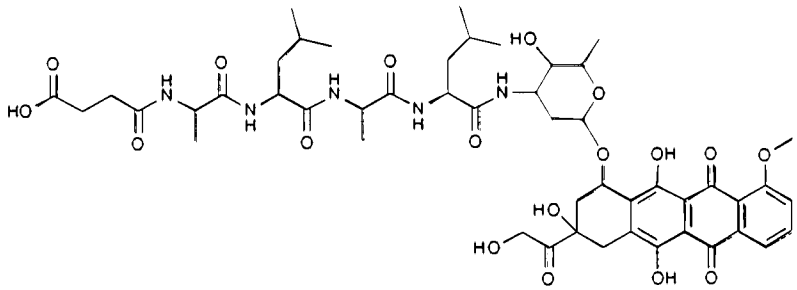


TA-HSSK(ivDde)LQL-Doxorubicin (0.250 g, 0.142 mmol, 1eq) was solubilised in dimethylformamide (10 mL) and hydrazine (0.2 mL). The solution was stirred for 20 minutes at room temperature and was directly quenched with HCL 1N at 4°C (added slowly due to exothermic reaction). The pH of the solution was checked and adjusted to pH 6. The resulting red solution was precipitated in *tert*-butyl methyl ether (200 mL) at 4°C. The resulting red solid was purified by chromatography with silica C18 (H_2O/acetonitrile 90:10 to 60:10). The fractions were analyzed by HPLC and the pure fractions were collected, pooled and lyophilized to afford the red solid.

Purity: 89 % (determined by HPLC at 214 nm).

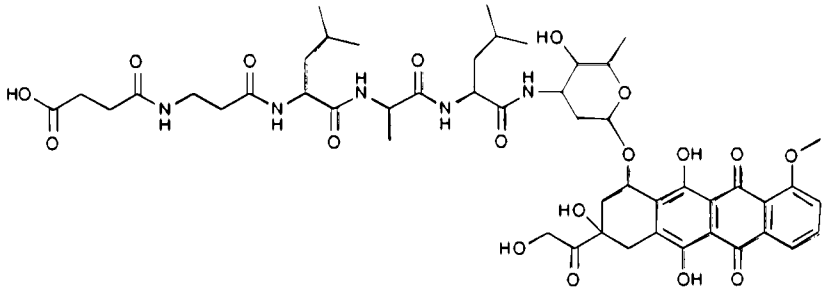
MS (ES^+): 1550 $[MH]^+$

I.2 Synthesis of succinyl-ALAL-doxorubicin



Succinyl-ALAL-doxorubicin was generated as previously described by Fernandez AM *et al.*, J Med Chem 44:3750-3753 (2001) [27].

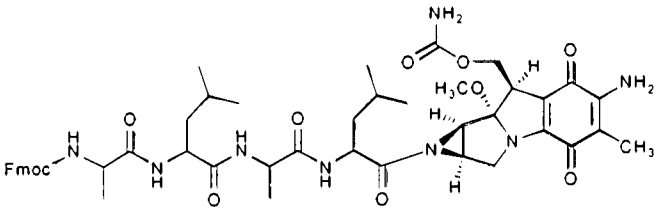
I.3 Synthesis of succinyl-βALAL-doxorubicin



Succinyl-βALAL-doxorubicin was generated in a similar manner to that described for succinyl-ALAL-doxorubicin.

I.4 Method of synthesis of TA-ALAL-mitomycin C

I.4.1 Synthesis of Fmoc-ALAL-mitomycin C

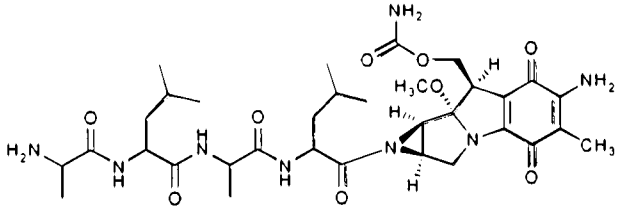


Mitomycin C (0.5 g, 1.5 mmol, 1 eq) was dissolved in dimethylformamide (20 mL) and water (5 mL) at +4°C. Fmoc-ALAL-OH (1.82 g, 3 mmol, 2 eq) was dissolved in dimethylformamide (25 mL) and added to the previous solution, followed by *O*-(7-azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (1.08 g, 2.84 mmol, 1.9 eq) in dichloromethane (5 mL). *N,N*-diisopropylethylamine (0.784 mL, 4.5 mmol, 3 eq) was added and the reaction mixture was stirred for 2h at +4°C. When mitomycin C was totally converted, dichloromethane (300 mL) was added to the reaction. The mixture was washed twice with a solution of NaCl 1M (100 mL), and once with a solution of aqueous citric acid 5% (100 mL). Residual water was removed by treatment with Na_2SO_4 and the organic phase was filtered and concentrated under vacuum. To the residual dimethylformamide of the concentrated organic layer dichloromethane (40 mL) was added and the mixture was precipitated using *tert*-butyl methyl ether (200 mL) at +4°C under agitation. The precipitate was filtered and dried. The purple precipitate was solubilised in a mixture of dichloromethane/methanol (98:2 v/v). This solution was purified by flash-chromatography on silica (dichloromethane 100% to dichloromethane/methanol 96:4 v/v). The recovered fractions were pooled and concentrated by rotary evaporation.

Purity: 94% (determined by HPLC at 214 nm).

MS (ES^+): 925 $[MH]^+$

I.4.2 Synthesis of NH_2-ALAL-mitomycin C

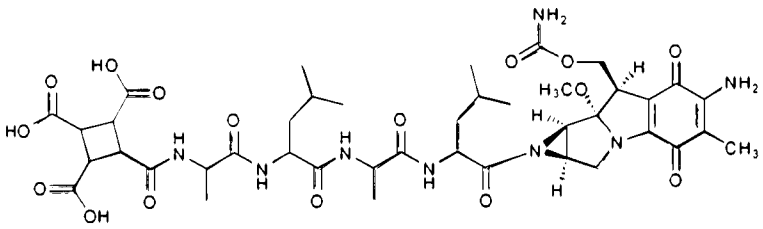


Fmoc-ALAL-mitomycin C (1 g, 1.08 mmol, 1 eq) was dissolved in dimethylformamide (22 mL) and piperidine (5.34 mL, 54 mmol, 50 eq) was added. The solution was stirred 90 seconds at room temperature and directly neutralized at +4°C with a cold solution of HCl 1N (9.0 mL, 54 mmol, 50 eq to obtain a pH of 7.4). The solution was stirred 10 min at +4°C and precipitated in *tert*-butyl methyl ether (450 mL) at +4°C. The precipitate was dissolved in water (400 mL) and the pH of the aqueous layer was adjusted at 8.8-9.0 with a solution of NaOH 1M. Then ALAL-mitomycin C was extracted 5 times with dichloromethane (300 mL). The organic layers were pooled, dried over Na_2SO_4, filtered and concentrated under vacuum to obtain a purple solid.

Purity: 94% (determined by HPLC at 214 nm).

MS (ES^+): 702 $[MH]^+$

I.4.3 Synthesis of TA-ALAL-mitomycin C



NH_2-ALAL-mitomycin C (0.4 g, 0.57 mmol, 1 eq) was dissolved in dimethylformamide (35 mL) at +4°C. *N*,*N*-diisopropylethylamine (0.497 mL, 2.85 mmol, 5 eq) was added followed by 1,2,3,4-cyclobutanetetracarboxylic acid (1.057 g, 4.55 mmol, 8 eq). Finally *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium hexafluorophosphate (0.323 g, 0.85 mmol, 1.5 eq) was added and the reaction mixture was stirred at +4°C for 45 minutes. When ALAL-mitomycin C was totally converted, the reaction mixture was precipitated using *tert*-butyl methyl ether (700 mL) at +4°C. After filtration and drying, the precipitate was dissolved in a solution of NaOH 1M and then purified by preparative

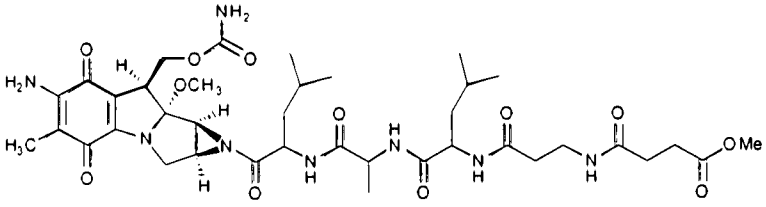
HPLC. The recovered fractions were pooled and the solution was lyophilized to obtain a purple solid.

Purity: 90.6% (determined by HPLC at 214 nm).

MS (ES^+): 939 $[M+Na]^+$

I.5 Method of synthesis of succinyl-βALAL-mitomycin C

I.5.1 Synthesis of OMe-succinyl-βALAL-mitomycin C

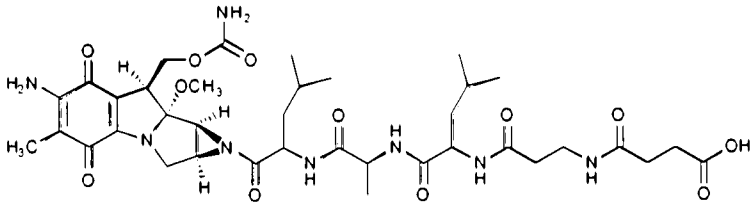


Mitomycin C (MMC, 0.400 g, 1.20 mmol, 1 eq) and OMe-succinyl-βALAL-OH (1.200 g, 2.40 mmol, 2 eq) were dissolved in dimethylformamide (15 mL) at +4°C. *N,N*-diisopropylethylamine (0.417 mL, 2.40 mmol, 2 eq) was added and the reaction mixture was stirred for 10 minutes at +4°C. A solution of 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (0.57 g, 1.38 mmol, 1.15 eq) in dimethylformamide (5 mL) was added dropwise and the reaction mixture was kept at +4°C. The reaction mixture was stirred at +4°C for 3 hours. Dichloromethane (60 mL) was added to the reaction. The organic layer was washed with a solution of NaCl 1M (35 mL) 4 times. The organic layer was concentrated under vacuum. Dichloromethane (10 mL) was added to the residual dimethylformamide of the concentrated organic layer and the solution was precipitated using *tert*-butyl methyl ether (100 mL) at +4°C. The precipitate was filtered and dried. The resulting solid was dissolved in a mixture of dichloromethane/methanol (95:5 v/v). The solution was purified by flash-chromatography (dichloromethane 100% v/v to dichloromethane/methanol 90:10 v/v). The recovered fractions were pooled and concentrated by rotary evaporation.

Purity: 95.4% (determined by HPLC at 214 nm).

MS (ES^+): 839 $[M+Na]^+$

I.5.2 Synthesis of succinyl-βALAL-mitomycin C



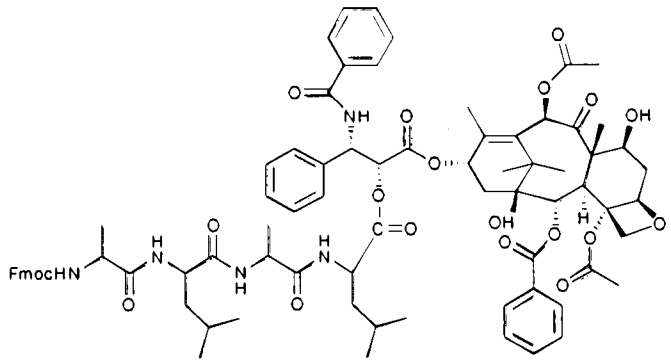
Candida antarctica lipase B cross-linked enzyme aggregate (CALB-CLEA, CLEA Technologies) (0.2 g) was washed 3 times with water (5 mL), and added in water (5 mL) to OMe-succinyl-βALAL-mitomycin C. This mixture was stirred at +37°C overnight and filtered. The aqueous filtrate was purified by preparative HPLC. The recovered fractions were pooled and lyophilized.

Purity: 94 % (determined by HPLC at 214 nm).

MS (ES^+): 825 $[M+Na]^+$

I.6 Method of synthesis of TA-ALAL-paclitaxel

I.6.1 Synthesis of Fmoc-ALAL-paclitaxel

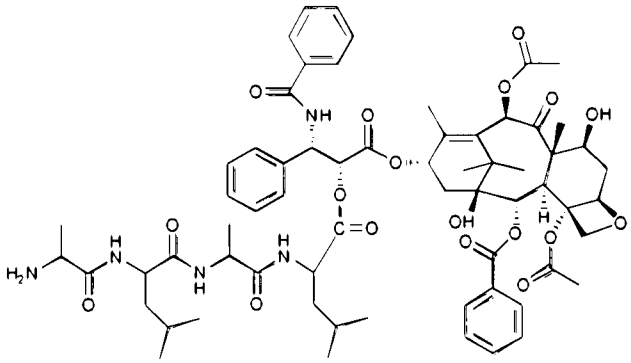


Paclitaxel (0.214 g, 0.251 mmol, 1 eq) was dissolved in dichloromethane (14 mL) at +4°C. 4-Dimethylaminopyridine (0.184 g, 1.504 mmol, 6 eq) was added followed by a solution of Fmoc-ALAL-OH (0.460 g, 0.752 mmol, 3 eq) in dimethylformamide (4 mL). The solution was stirred at +4°C for 10 minutes then *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (0.145 g, 0.752 mmol, 3 eq) was added. The solution was stirred at +4°C for 10 minutes, then 3 hours at room temperature. Dichloromethane was added (100 mL) and the solution was washed twice with NaCl 1M (50 mL) and twice with aqueous citric acid 5% (50 mL). The organic layer was concentrated under vacuum, dissolved in a minimum of dichloromethane and precipitated using *tert*-butyl methyl ether at +4°C. The pale yellow solid was filtered and dried.

Purity: 90% (determined by HPLC at 214 nm).

MS (ES^+): 1444 $[MH]^+$

I.6.2 Synthesis of NH_2-ALAL-paclitaxel

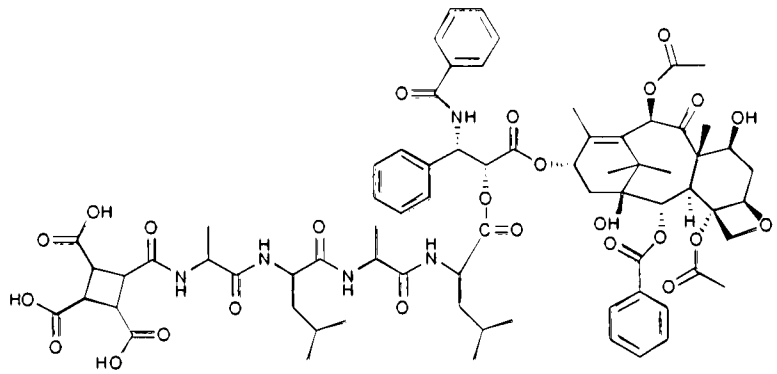


Fmoc-ALAL-paclitaxel (362 mg, 0.251mmol, 1 eq) was dissolved in dimethylformamide (5 mL), and piperidine (1.25 mL, 12.55 mmol, 50 eq) was added. The solution was stirred at room temperature for 90 seconds and directly quenched at +4°C with HCl 6N (2.1 mL, 12.55 mmol, 50 eq). Dichloromethane (50 mL) was added and the solution was washed three times with H_2O (50 mL) and once with NaCl 1M (50 mL). The organic layer was evaporated to obtain a pale yellow solid.

Purity: 90% (determined by HPLC at 214 nm)..

MS (ES^+): 1222 $[MH]^+$

I.6.3 Synthesis of TA-ALAL-paclitaxel



NH_2-ALAL-paclitaxel (285 mg, 0.233 mmol, 1eq) generated in the previous step was dissolved in dimethylformamide (8 mL) and *N,N*-diisopropylethylamine (0.125 mL, 0.7 mmol, 3 eq) was added. The solution was stirred 5 minutes at room temperature then 1,2,3,4-cyclobutanetetracarboxylic acid (0.540 g, 2.33 mmol, 10 eq) was added followed by 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate

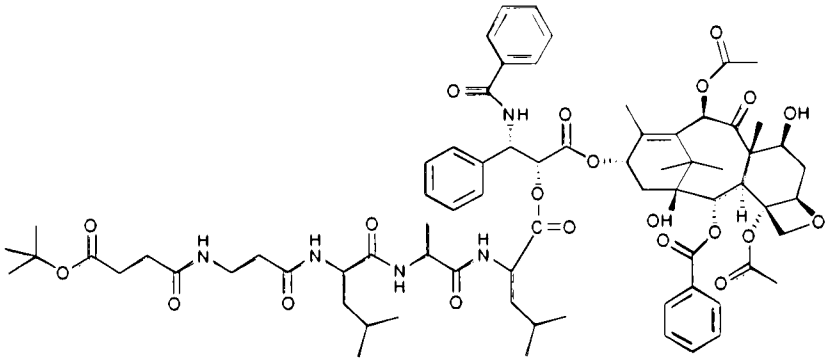
(0.155 g, 0.377 mmol, 1.5 eq). The reaction was stirred 1 hour at room temperature then the crude conjugate was dissolved in phosphate buffer (pH 7.1, 1M) (3 mL) and a sufficient volume of acetonitrile was added to maintain a clear solution. The solution was purified by preparative HPLC. The recovered HPLC fractions were pooled and lyophilized.

Purity: 99.2% (determined by HPLC at 214 nm).

MS (ES^+): 1434 $[MH]^+$

I.7 Method of synthesis of succinyl-βALAL-paclitaxel

I.7.1 Synthesis of *t*Bu-succinyl-βALAL-paclitaxel

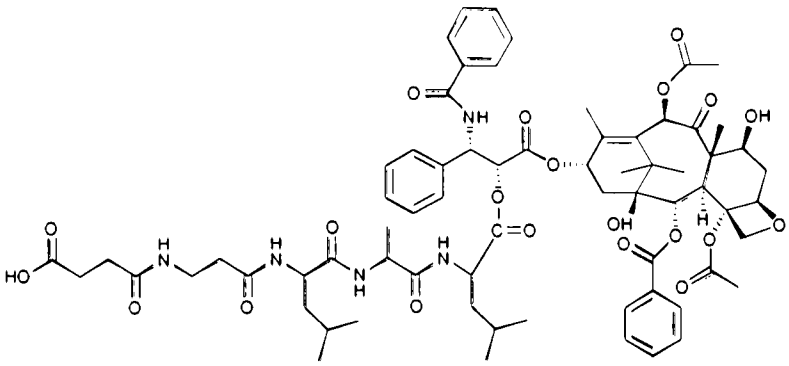


Paclitaxel (20 mg, 0.023 mmol, 1 eq) was dissolved in dichloromethane (0.5 mL) at +4°C. To this solution was added 4-dimethylaminopyridine (17 mg, 0.141 mmol, 6 eq). tBu-succinyl-βALAL-OH (38 mg, 0.070 mmol, 3 eq) was added at +4°C followed by *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (13 mg, 0.070 mmol, 3 eq). The solution was stirred at +4°C for 10 minutes, then 30 minutes at room temperature. Dichloromethane (5 mL) was added and the organic layer was washed twice with citric acid 5% (2 mL), twice with $NaHCO_3$ 1M (2 mL) and twice with NaCl 1M (2 mL). The organic layer was evaporated under vacuum until only a residual amount of solvent remained. The *t*Bu-succinyl-βALAL-paclitaxel was precipitated using *tert*-butyl methyl ether (5 mL).

Purity: 86% (determined by HPLC at 214 nm).

MS (ES^+): 1378 $[MH]^+$

I.7.2 Synthesis of succinyl-βALAL-paclitaxel



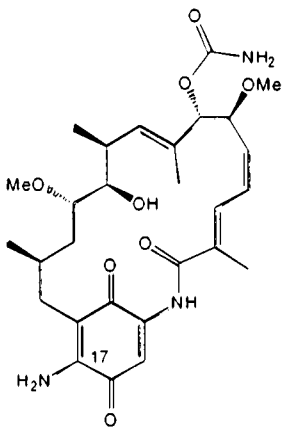
*t*Bu-succinyl-*β*ALAL-paclitaxel (5 mg, 0.036 mmol, 1eq) was dissolved in a solution of trifluoroacetic acid/dichloromethane/triisopropylsilane (10:20:0.5, 0.215 mL) and stirred for 20 minutes at room temperature. The solution was neutralized (pH 6) with *N,N*-diisopropylethylamine. The crude conjugate was purified by preparative HPLC and the recovered fractions were pooled and lyophilized.

Purity: 93% (determined by HPLC at 214 nm).

MS (ES^+): 1344 $[M+Na]^+$

I.8 Method of synthesis of TA-ALAL-geldanamycin

I.8.1 Synthesis of 17-aminogeldanamycin

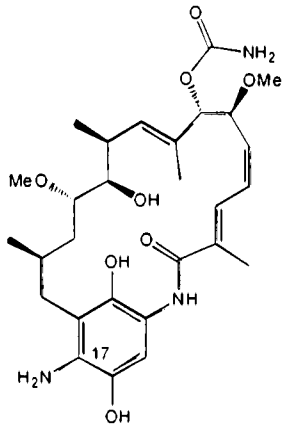


Geldanamycin (0.200 g, 0.36 mmol, 1 eq) was dissolved in 1,2-dichloroethane (9 mL). Ammonia 2N in methanol (9 mL, 18 mmol, 50 eq) was added and the solution was stirred at 50°C for 3 hours, until the yellow solution turned purple. The solvent was evaporated and the resulting oil purified by flash chromatography (DCM/MeOH 95:5). The recovered fractions were pooled and concentrated by rotary evaporation.

Purity: 98% (determined by HPLC at 214 nm).

MS (ES^+): 567 $[M+Na]^+$

I.8.2 Synthesis of 18,21-dihydro-17-aminogeldanamycin

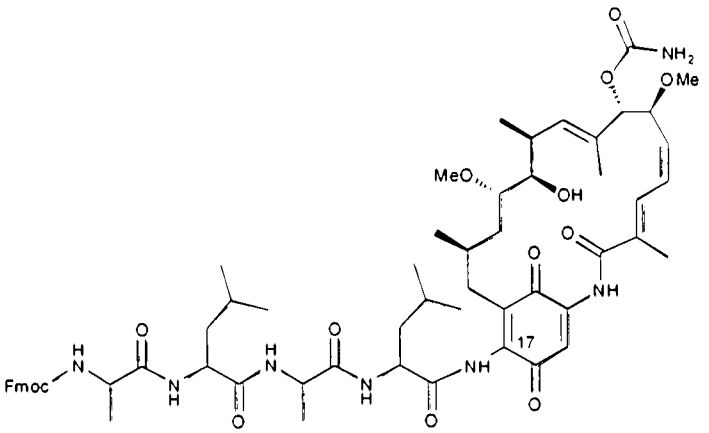


17-aminogeldanamycin (0.150 g, 0.28 mmol, 1 eq) was dissolved in ethyl acetate (3 mL) and an aqueous solution of 10% $Na_2S_2O_4$ (2.5 mL) was added. The solution was stirred vigorously until the solution turned yellow. Ethyl acetate (5 mL) and water (5 mL) were added and the aqueous layer was extracted 5 times with ethyl acetate. The combined organic layers were evaporated and the resulting yellow powder kept under argon at -20°C.

Purity: 95% (determined by HPLC at 214 nm).

MS (ES^+): 569 $[M+Na]^+$

I.8.3 Synthesis of 17-Fmoc-ALAL-aminogeldanamycin



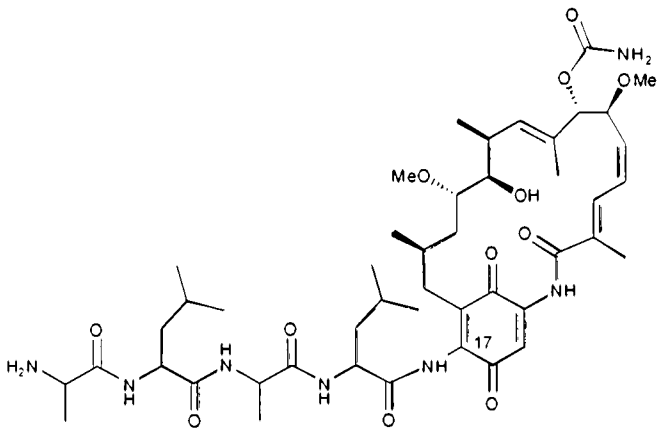
Fmoc-ALAL-OH (0.822 g, 1.35 mmol, 5 eq) and *N,N*-dicyclohyexylcarbodiimide (0.557 g, 2.7 mmol, 10 eq) were dissolved in dichloromethane (10 mL) under argon and

18,21-dihydro-17-aminogeldanamycin (0.150 g, 0.27 mmol, 1 eq) was added. The solution was stirred at room temperature under argon for 18 hours. *N,N*-diisoproylethylamine (54 μL, 0.31 mmol, 1.15 eq) was added followed by copper (II) sulfate (21.5 mg, 0.135 mmol, 0.5 eq) and methanol (0.383 mL). The solution was stirred at room temperature for 1 hour. Dichloromethane (5 mL) was added and the organic layer washed with HCl 1N (10 mL), $NaHCO_3$ 1M (10 mL) and water (10 mL). The organic layer was dried over $MgSO_4$, evaporated and the resulting oil purified by flash chromatography (DCM/MeOH 90:10). The recovered fractions were pooled and concentrated by rotary evaporation.

Purity: 92% (determined by HPLC at 214 nm).

MS (ES^+): 1156 $[M+Na]^+$

I.8.4 Synthesis of 17-NH_2-ALAL-aminogeldanamycin

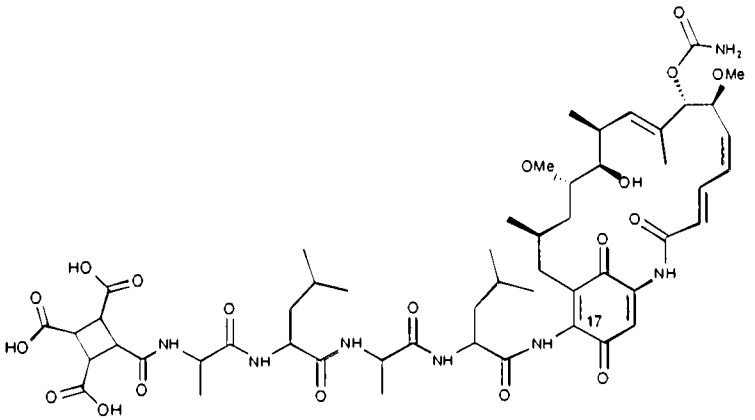


17-Fmoc-ALAL-aminogeldanamycin (0.215 mg, 0.191 mmol, 1 eq) was dissolved in dimethylformamide (3.8 mL) and piperidine (0.946 mL, 9.55 mmol, 50 eq) was added. The solution was stirred for 90 seconds at room temperature and directly neutralized at +4°C with a cold solution of HCl 6N (1.59 mL, 9.55 mmol, 50 eq) to obtain a pH 6. The crude was purified by preparative HPLC. The recovered fractions were pooled and lyophilized.

Purity: 92% (determined by HPLC at 214 nm).

MS (ES^+): 914 $[MH]^+$

I.8.5 Synthesis of 17-TA-ALAL-aminogeldanamycin (further called TA-ALAL-geldanamycin)



17-NH_2-ALAL-aminogeldanamycin (0.155 mg, 0.17 mmol, 1 eq) was dissolved in dimethylformamide (4 mL). *N,N*-diisopropylethylamine (0.148 mL, 0.85 mmol, 5 eq) was added followed by 1,2,3,4-cyclobutanetetracarboxylic acid (0.394 g, 1.7 mmol, 10 eq). After stirring for 5 minutes, *O*-(7-Azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (0.097 g, 0.255 mmol, 1.5 eq) was added. The solution was stirred for 30 minutes at room temperature and directly purified by preparative HPLC. The recovered HPLC fractions were pooled and lyophilized.

Purity: 98 % (determined by HPLC at 214 nm).

MS (ES^+): 1149 $[MNa]^+$

I.9 Method of synthesis of succinyl-βALAL-geldanamycin

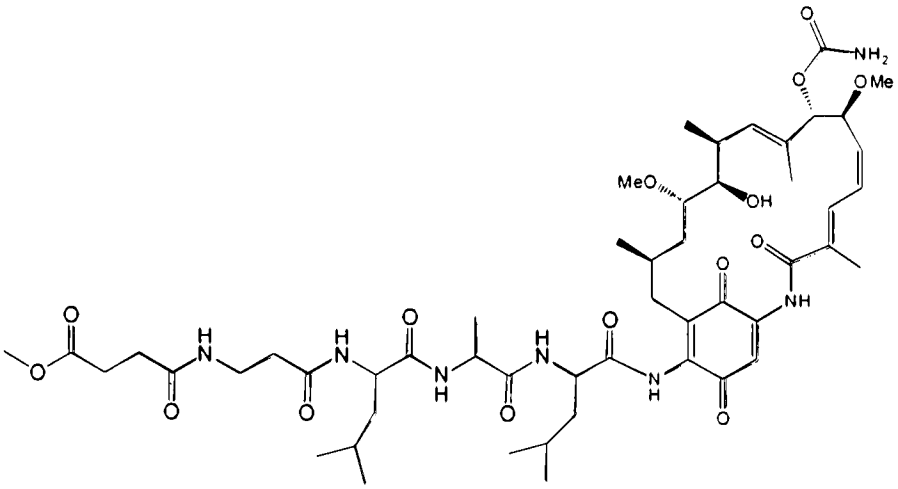
I.9.1 Synthesis of 17-aminogeldanamycin

Prepared as described in paragraph I.8.1.

I.9.2 Synthesis of 18,21-dihydro-17-aminogeldanamycin

Prepared as described in paragraph I.8.2

I.9.3 Synthesis of 17-OMe-succinyl-βALAL-aminogeldanamycin

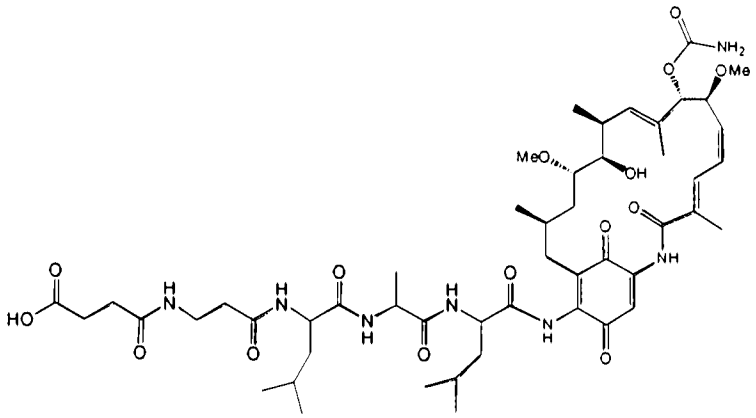


OMe-succinyl-βALAL-OH (0.340 g, 0.68 mmol, 5 eq) and *N,N'*-dicyclohyexylcarbodiimide (0.279 g, 1.35 mmol, 10 eq) were dissolved in dichloromethane (10 mL) under argon and 18,21-dihydro-17-aminogeldanamycin (0.075 g, 0.14 mmol, 1 eq) was added. The solution was stirred at room temperature under argon for 18 hours. *N,N*-diisoproylethylamine (27 μL, 0.15 mmol, 1.15 eq) was added followed by copper(II)sulfate (10.5 mg, 0.068 mmol, 0.5 eq) and methanol (190 μL). The solution was stirred at room temperature for 1 hour. Dichloromethane (3 mL) was added and the organic layer washed with HCl 1N (5 mL), $NaHCO_3$ 1M (5 mL) and water (5 mL). The organic layer was dried over $MgSO_4$, evaporated and the resulting oil purified by flash chromatography (DCM/MeOH 90:10). The recovered fractions were pooled and concentrated by rotary evaporation.

Purity: 85% (determined by HPLC at 214 nm).

MS (ES^+): 1051 $[M+Na]^+$

I.9.4 Synthesis of 17-succinyl-βALAL-aminogeldanamycin (further called succinyl-βALAL-geldanamycin)



Candida antarctica lipase B cross-linked enzyme aggregate (CALB-CLEA, CLEA Technologies) (0.1 g) was washed 3 times with water (3 mL), and added in water (3 mL) to 17-OMe-succinyl-βALAL-aminogeldanamycin (0.05 g, 0.049 mmol). This mixture was stirred at +37°C overnight and filtered. The aqueous filtrate was purified by preparative HPLC. The recovered fractions were pooled and lyophilized.

Purity: 91 % (determined by HPLC at 214 nm).

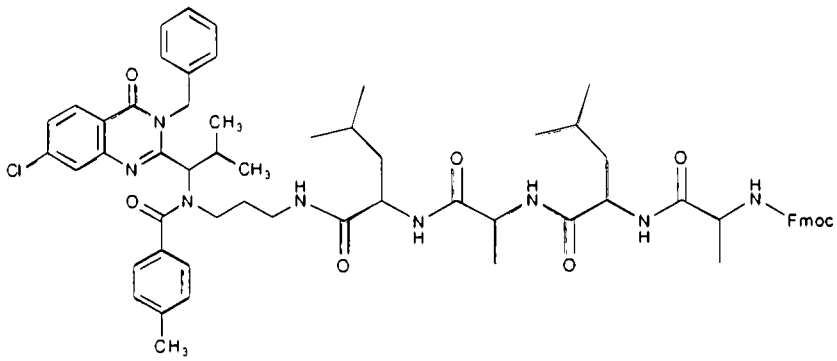
MS (ES^+): 1026 $[M+Na]^+$

I.10 Method of synthesis of TA-ALAL-ispinesib

I.10.1 Synthesis of ispinesib mesylate

Synthesis was performed as described in WO 03/070701 [25].

I.10.2 Synthesis of Fmoc-ALAL-ispinesib



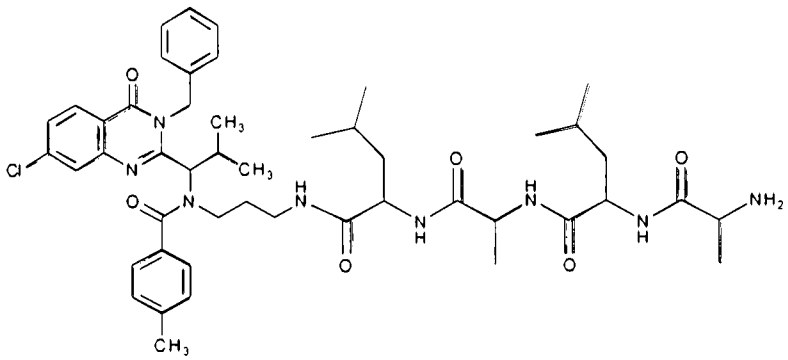
Ispinesib mesylate (20 mg, 0.033 mmol, 1 eq) was dissolved in dimethylformamide (0.4 mL). *N,N*-diisopropylethylamine (17.9 µL, 0.123 mmol, 3 eq) was added followed by Fmoc-ALAL-OH (30 mg, 0.049 mmol, 1.5 eq). After stirring for 2 minutes, 2-(6-chloro-

1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (19 mg, 0.046 mmol, 1.4 eq) was added. The solution was stirred for 1 hour at room temperature. Dichloromethane (1 mL) was added and the organic layer washed with NaCl 1M (0.5 mL) and aqueous citric acid 5% (0.5 mL). The organic layer was dried over $MgSO_4$ and evaporated to obtain a yellow oil.

Purity: 91 % (determined by HPLC at 214 nm).

MS (ES^+): 1130 $[MNa]^+$

I.10.3 Synthesis of NH_2-ALAL-ispinesib

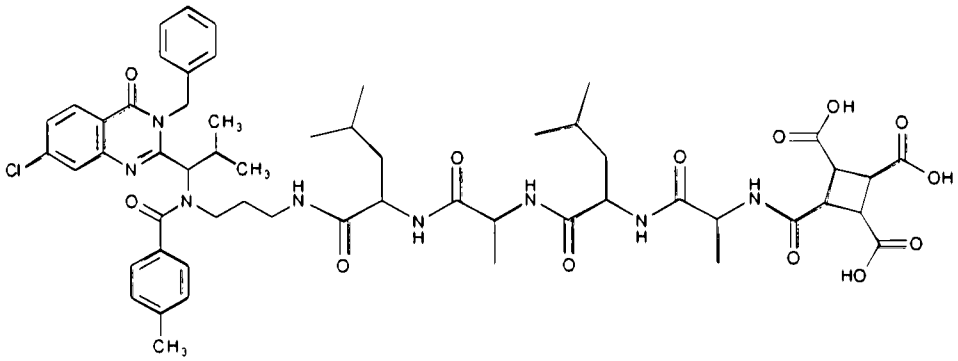


Fmoc-ALAL-ispinesib (20 mg, 0.018 mmol, 1 eq) was dissolved in dimethylformamide (0.355 mL) and piperidine (0.09 mL, 0.90 mmol, 50 eq) was added. The solution was stirred for 3 minutes at room temperature and directly neutralized at +4°C with a cold solution of HCl 6N (0.15 mL, 0.90 mmol, 50 eq) to obtain a pH of 6. Dichloromethane (1 mL) was added and the organic layer washed with aqueous citric acid 5% (0.5 mL), NaCl 1M (0.5 mL). The organic layer was dried over $MgSO_4$ and evaporated to obtain a yellow oil.

Purity: 92 % (determined by HPLC at 214 nm).

MS (ES^+): 908 $[MNa]^+$

I.10.4 Synthesis of TA-ALAL-ispinesib



NH_2-ALAL-ispinesib (21 mg, 0.024 mmol, 1 eq) was dissolved in dimethylformamide (0.5 mL). *N,N*-diisopropylethylamine (12.6 µL, 0.072 mmol, 3 eq) was added followed by 1,2,3,4-cyclobutanetetracarboxylic acid (55 mg, 0.24 mmol, 10 eq). After stirring 5 minutes, *O*-(7-Azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (13.68 mg, 0.036 mmol, 1.5 eq) was added. The solution was stirred 1 hour at room temperature. Dichloromethane (1 mL) was added and the organic layer washed twice with NaCl 1M and twice with aqueous citric acid 5% (5 mL). The organic layer was dried over $MgSO_4$ and evaporated to obtain a white solid. The crude solid was suspended in dimethylformamide (1 mL), acetonitrile (1 mL) and water (4 mL) and then purified by preparative HPLC. The recovered HPLC fractions were pooled and lyophilized.

Purity: 95 % (determined by HPLC at 214 nm).

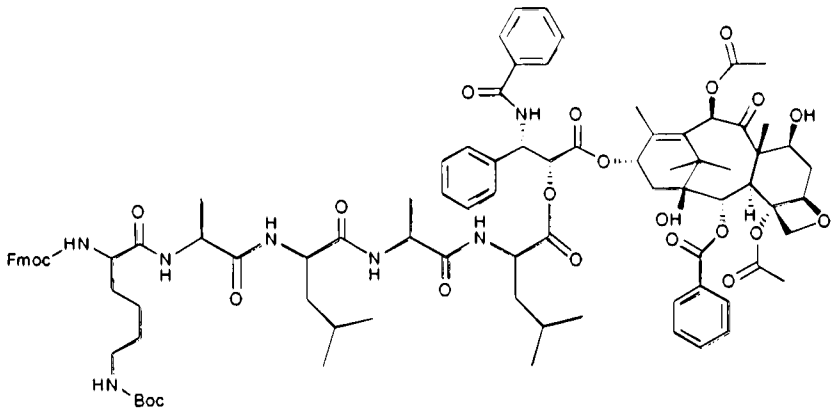
MS (ES^+): 1100 $[MH]^+$

I.11 Method of synthesis of TA-KALAL-paclitaxel

I.11.1 Synthesis of NH_2-ALAL-paclitaxel

Prepared as described in paragraph I.6.2

I.11.2 Synthesis of Fmoc-K(Boc)ALAL-paclitaxel

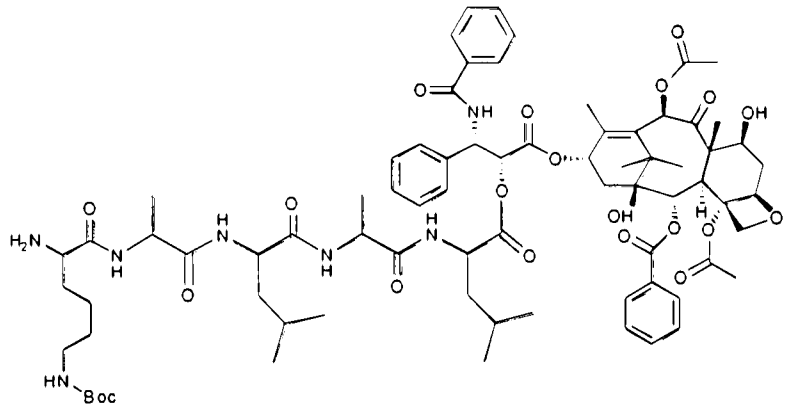


NH_2-ALAL-paclitaxel (50 mg, 0.041 mmol, 1 eq) was dissolved in dimethylformamide (0.55 mL). *N,N*-diisopropylethylamine (22 µL, 0.123 mmol, 3 eq) was added followed by Fmoc-Lysine(Boc)-OH (0.038 g, 0.082 mmol, 2 eq). After stirring 5 minutes, 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (0.032 g, 0.078 mmol, 1.9 eq) was added. The solution was stirred 2 hours at room temperature. Dichloromethane (5 mL) was added and the organic layer washed with NaCl 1M (5 mL) and aqueous citric acid 5% (5 mL). The organic layer was dried over $MgSO_4$ and evaporated to obtain a yellow oil.

Purity: 89 % (determined by HPLC at 214 nm).

MS (ES+): 1646 [MNa]+

I.11.3 Synthesis of NH_2-K(Boc)ALAL-paclitaxel



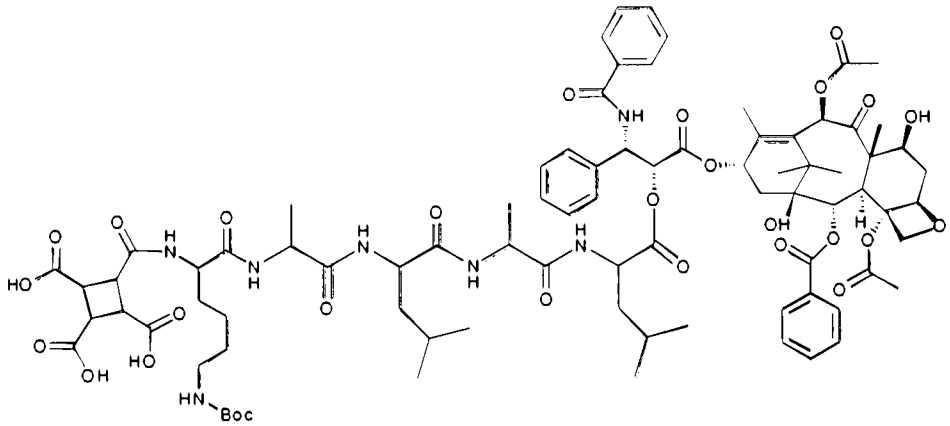
Fmoc-K(Boc)ALAL-paclitaxel (47 mg, 29 µmol, 1 eq) was dissolved in dimethylformamide (0.565 mL) and piperidine (0.141 mL, 1.43 mmol, 50 eq) was added. The solution was stirred 90 seconds at room temperature and directly neutralized at +4°C

with a cold solution of HCl 6N (0.238 mL, 1.43 mmol, 50 eq) to obtain a pH of 6. The crude was purified by flash chromatography (dichloromethane/ methanol 90:10).

Purity: 89 % (determined by HPLC at 214 nm).

MS (ES^+): 1451 $[MH]^+$

I.11.4 Synthesis of TA-K(Boc)ALAL-paclitaxel

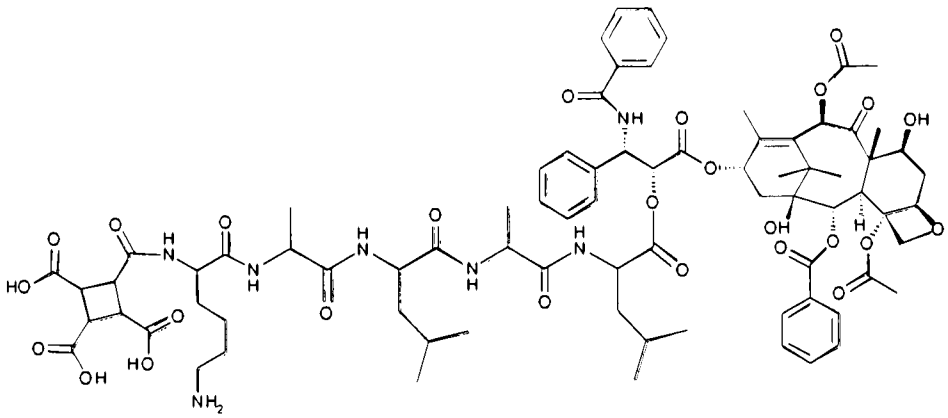


NH_2-K(Boc)ALAL-paclitaxel (35 mg, 0.024 mmol, 1 eq) was dissolved in dimethylformamide (0.5 mL). *N,N*-diisopropylethylamine (21 µL, 0.12 mmol, 5 eq) was added followed by 1,2,3,4-cyclobutanetetracarboxylic acid (55 mg, 0.24 mmol, 10 eq). After stirring 5 minutes, *O*-(7-Azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (13.68 mg, 0.036 mmol, 1.5 eq) was added. The solution was stirred 2 hours at room temperature. Dichloromethane (5 mL) was added and the organic layer washed with NaCl 1M and aqueous citric acid 5% (5 mL). The organic layer was dried over $MgSO_4$ and evaporated to obtain a yellow oil.

Purity: 88 % (determined by HPLC at 214 nm).

MS (ES^+): 1686 $[MNa]^+$

I.11.5 Synthesis of TA-KALAL-paclitaxel



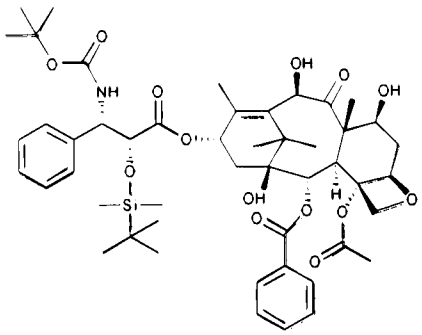
TA-K(Boc)ALAL-paclitaxel (26 mg, 0.015 mmol, 1 eq) was solubilised in dichloromethane (1 mL) and trifluoroacetic acid (1 mL) was added. The solution was stirred at room temperature for 5 minutes and directly quenched at pH 5 with NaOH 3M. The crude solution was then purified by preparative HPLC. The recovered HPLC fractions were pooled and lyophilized.

Purity: 92 % (determined by HPLC at 214 nm).

MS (ES^+): 1564 $[MH]^+$

I.12 Method of synthesis of 10-*O*-(carbamate(TA-ALAL)-*N*-(2-methylaminoethyl)amide))docetaxel

I.12.1 Synthesis of 2'-*O*-(*tert*-butyldimethylsilyl)docetaxel



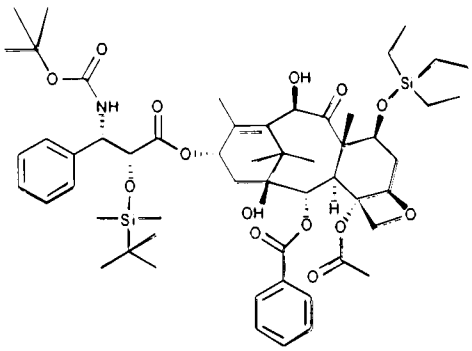
Imidazole (52 mg, 0.74 mmol, 12 eq) and *tert*-butyldimethylsilylchloride (95 mg, 0.63 mmol, 10 eq) was dissolved in dimethylformamide (0.4 mL). The solution was stirred at room temperature for 45 minutes. A solution of docetaxel (50 mg, 0.061 mmol, 1 eq) in dimethylformamide (0.4 mL) was added. The solution was stirred for 3 hours at room temperature. Dichloromethane (2 mL) was added and the solution was washed twice with a saturated aqueous solution of NH_4Cl (1 mL). The organic layer was dried over Na_2SO_4

and purified by flash chromatography (dichloromethane/methanol 96:4). The recovered fractions were pooled and concentrated under vacuum.

Purity: 97% (determined by HPLC at 214 nm).

MS (ES^+): 944 [MNa+]

I.12.2 Synthesis of 2'-*O*-(*tert*-butyldimethylsilyl)-7-*O*-(triethylsilyl)docetaxel

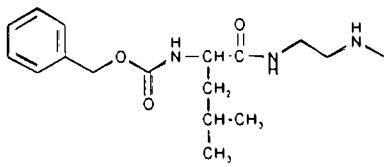


2'-*O*-(*tert*-butyldimethylsilyl)docetaxel (50 mg, 0.054 mmol, 1 eq) was dissolved in acetonitrile/dichloromethane (50:50, 1 mL). The solution was stirred at +60°C for 45 minutes. At the same time, imidazole (55 mg, 0.81 mmol, 15 eq) and chlorotriethylsilane (126 mg, 0.84 mmol, 15.5 eq) were heated at 60°C in acetonitrile/dichloromethane (50:50, 0.75 mL). Both solutions were mixed quickly and stirred at 60°C for 90 minutes. After chilling at room temperature, dichloromethane (2 mL) was added and the solution was washed twice with a saturated aqueous solution of NH_4Cl (1 mL). The organic layer was concentrated under vacuum, dissolved in dichloromethane (2 mL) and purified by flash chromatography (heptane/ethylacetate 80:20). The recovered fractions were pooled and concentrated under vacuum.

Purity: 90% (determined by HPLC at 214 nm).

MS (ES^+): 1059 [MNa+]

I.12.3 Synthesis of *N*-CbZ-Leu-*N*-(2-methylaminoethyl)amide



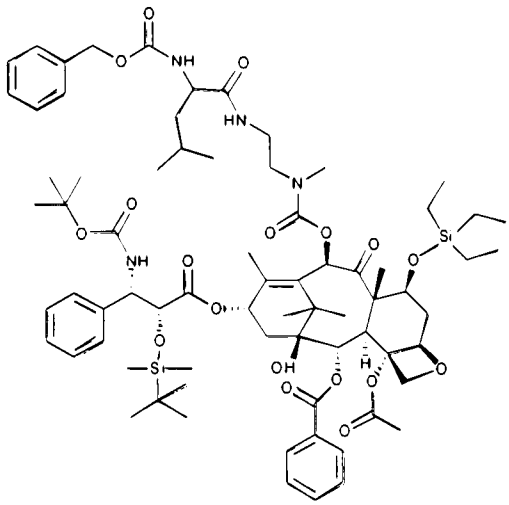
N-Methylethylenediamine (100 mg, 1.15 mmol, 5 eq) and *N,N*-diisopropylethylamine (0.12 mL, 0.69 mmol, 3 eq) was dissolved in dimethylformamide (1 mL). *N*-CbZ-

Leucine (61 mg, 0.23 mmol, 1 eq) in dimethylformamide (0.1 mL) was added, followed by a solution of 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (140 mg, 0.035 mmol, 1.5 eq) in dimethylformamide (0.2 mL). This mixture was stirred at room temperature for 3 hours. Dichloromethane (3 mL) was added and the crude product was purified by flash chromatography (dichloromethane /methanol 90:10). The recovered fractions were pooled and concentrated under vacuum.

Purity: 90% (determined by HPLC at 214 nm).

MS (ES^+): 322 [MH+]

I.12.4 Synthesis of 2'-*O*-(*tert*-butyldimethylsilyl)-7-*O*-(triethylsilyl)-10-*O*-(carbamate-*N*-CbZ-Leu-*N*-(2-methylaminoethyl)amide)docetaxel



2'-*O*-(*tert*-butyldimethylsilyl)-7-*O*-(triethylsilyl)docetaxel (40 mg, 0.039 mmol, 1 eq) was dissolved in dichloromethane (0.5 mL) at room temperature and carbonyldiimidazole (31.6 mg, 0.195 mmol, 5 eq) was added. The solution was stirred at room temperature under argon for 18 hours. Dichloromethane (1 mL) was added and the solution was washed twice with H_2O (1 mL). The organic layer was dried over Na_2SO_4 and then concentrated under vacuum.

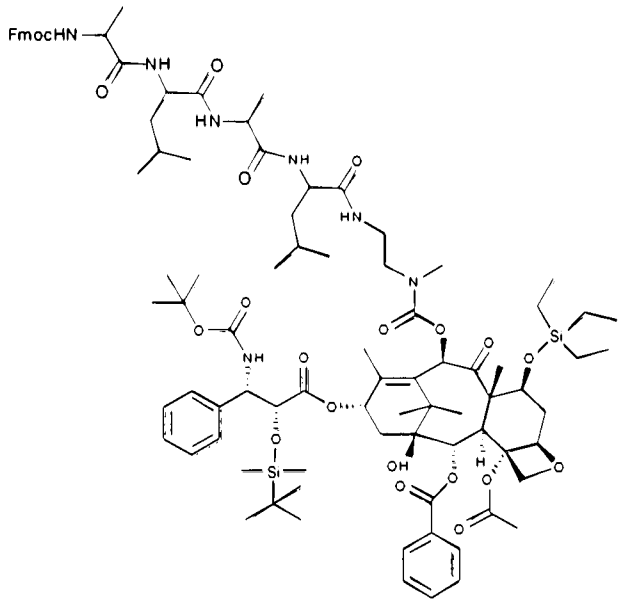
The resulting product (30 mg, 0.027mmol, 1 eq) was dissolved in *tert*-butanol (0.5 mL) and warmed at +90°C. 4-Dimethylaminopyridine (6.6 mg, 0.054 mmol, 2 eq) and *N*-CbZ-Leu-*N*-(2-methylaminoethyl)amide (43.4 mg, 0.135 mmol, 5 eq) were added and the solution was stirred at +90°C for 3 hours. Dichloromethane (2 mL) was added and the solution was washed twice with a saturated aqueous solution of NH_4Cl (1 mL). The

organic layer was concentrated under vacuum, dissolved in a minimum of dichloromethane and purified by flash chromatography. The recovered fractions were pooled and concentrated under vacuum.

Purity: 90% (determined by HPLC at 214 nm).

MS (ES$^+$): 1384 [MH]$^+$

I.12.5 Synthesis of 2'-*O*-(*tert*-butyldimethylsilyl)-7-*O*-(triethylsilyl)-10-*O*-(carbamate(Fmoc-ALAL-*N*-(2-methylaminoethyl)amide))docetaxel



2'-*O*-(*tert*-butyldimethylsilyl)-7-*O*-(triethylsilyl)-10-*O*-(carbamate-*N*-CbZ-Leu-*N*-(2-methylaminoethyl)amide)docetaxel (20 mg, 0.014 mmol, 1 eq) was dissolved in 4% formic acid in methanol (0.5 mL). Palladium on activated carbon (6 mg) was added and the suspension was stirred under argon for 30 minutes. The suspension was filtered on celite and then evaporated under vacuum. The resulting oil was dissolved in dichloromethane (1 mL) and purified by flash chromatography. The recovered fractions were pooled and concentrated under vacuum.

Purity: 92% (determined by HPLC at 214 nm).

MS (ES$^+$): 1249 [MH]$^+$

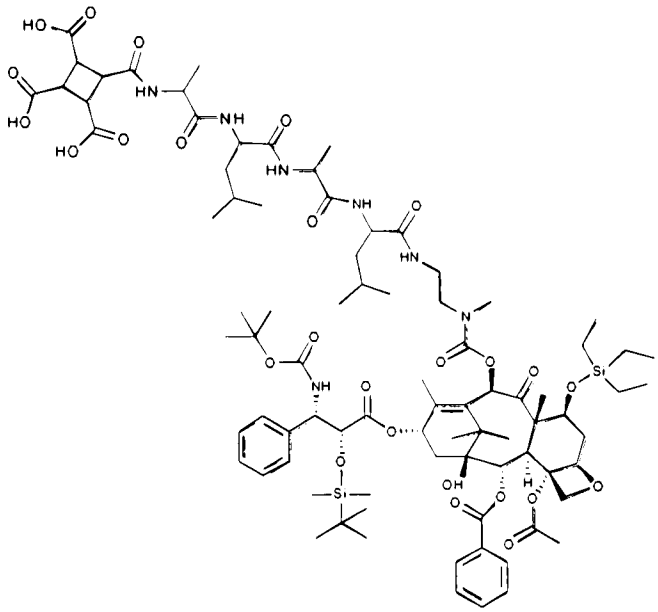
2'-*O*-(*tert*-butyldimethylsilyl)-7-*O*-(triethylsilyl)-10-*O*-(carbamate-Leu-*N*-(2-methyl aminoethyl)amide)docetaxel (18 mg, 0.014 mmol, 1 eq) was dissolved in

dichloromethane (0.5 mL) at +4°C. *N,N*-Diisopropylethylamine (8.8 µL, 0.05mmol, 3.5 eq) was added, followed by a solution of Fmoc-ALA-OH (13.9 mg, 0.028 mmol, 2eq) in dimethylformamide (0.2 mL). The solution was stirred at +4°C for 10 minutes before the addition of a solution of 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (11.8 mg, 0.021 mmol, 1.5 eq) in dimethylformamide (0.2 mL). The solution was stirred at +4°C for 10 minutes, then 45 minutes at room temperature. Dichloromethane (5 mL) was added and the solution was washed with NaCl 1M (2 mL) and with aqueous citric acid 5% (2 mL). The organic layer was concentrated under vacuum.

Purity: 70% (determined by HPLC at 214 nm).

MS (ES^+): 1498 $[MH]^+$

I.12.6 Synthesis of 2'-*O*-(*tert*-butyldimethylsilyl)-7-*O*-(triethylsilyl)-10-*O*-(carbamate(TA-ALAL)-*N*-(2-methylaminoethyl)amide)docetaxel



2'-*O*-(*tert*-butyldimethylsilyl)-7-*O*-(triethylsilyl)-10-*O*-(carbamate(Fmoc-ALAL-*N*-(2-methylaminoethyl)amide))docetaxel (20 mg, 0.011 mmol, 1 eq) was dissolved in dimethylformamide (0.136 mL), and piperidine (0.034 mL, 0.44 mmol, 40 eq) was added. The solution was stirred at room temperature for 5 minutes and directly quenched at +4°C with HCl 6N (0.057 mL, 0.44 mmol, 40 eq). Dichloromethane (10 mL) was

added and the solution was washed twice with H_2O (10 mL). The organic layer was evaporated under vacuum to obtain a white solid.

Purity: 90% (determined by HPLC at 214 nm).

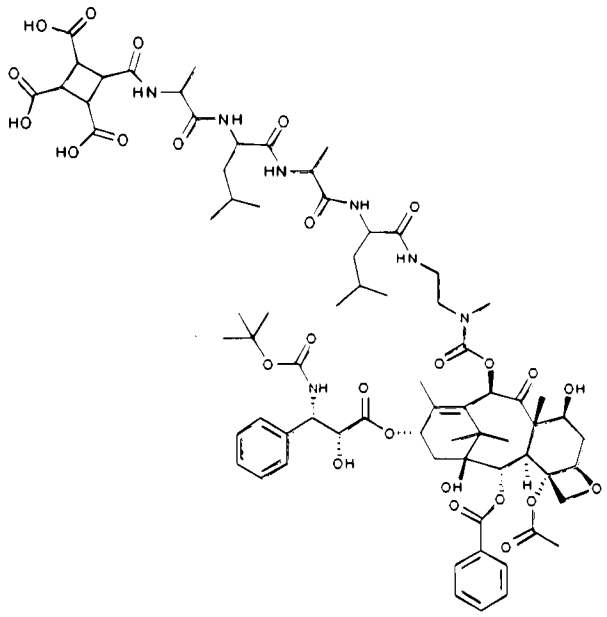
MS (ES^+): 1504 $[MH]^+$

2'-*O*-(*tert*-butyldimethylsilyl)-7-*O*-(triethylsilyl)-10-*O*-(carbamate(ALAL-*N*-(2-methylaminoethyl)amide))docetaxel (16.5 mg, 0.011 mmol, 1 eq) was dissolved in dimethylformamide (8 mL) and *N,N*-diisopropylethylamine (6.7 μL, 0.039 mmol, 3.5 eq) was added. The solution was stirred 5 minutes at room temperature then 1,2,3,4-cyclobutanetetracarboxylic acid (0.02 mg, 0.088 mmol, 8 eq) was added followed by 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (0.006 mg, 0.017 mmol, 1.5 eq). The solution was stirred at room temperature for 1 hour. The crude conjugate was dissolved in acetonitrile (0.3 mL) and H_2O (0.3 mL) was added. NaOH 0.1 M was added dropwise until pH 6.5. The solution was purified by preparative HPLC. The recovered HPLC fractions were pooled and lyophilized.

Purity: 99.2% (determined by HPLC at 214 nm).

MS (ES^+): 1719 $[MH]^+$

I.12.7 Synthesis of 10-*O*-(carbamate(TA-ALAL)-*N*-(2-methylaminoethyl)amide) docetaxel



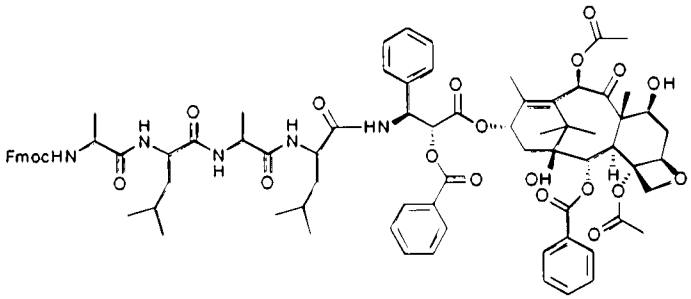
2'-*O*-(*tert*-butyldimethylsilyl)-7-*O*-(triethylsilyl)-10-*O*-(carbamate(TA-ALAL)-*N*-(2-methylaminoethyl)amide)docetaxel (15 mg, 0.009 mmol, 1 eq) was dissolved in acetonitrile (0.3 ml) and pyridine (0.3 ml) and the solution cooled at +4°C. HF/pyridine (70:30, 0.263 ml) was added dropwise. The resulting solution was stirred for 3 hours allowing the temperature to rise to room temperature. Dichloromethane (2 mL) was added and a saturated aqueous solution of $NaHCO_3$ (10 mL) was added carefully. The organic layer was extracted, dried over Na_2SO_4, filtered, evaporated under vacuum and dissolved in water (1.4 mL) and acetonitrile (0.6 mL). The solution was purified by preparative HPLC. The recovered HPLC fractions were pooled and lyophilized.

Purity: 99.2% (determined by HPLC at 214 nm).

MS (ES^-): 1488 $[MH]^-$

I.13 Method of synthesis of TA-ALAL-isotaxel

I.13.1 Synthesis of Fmoc-ALAL-isotaxel

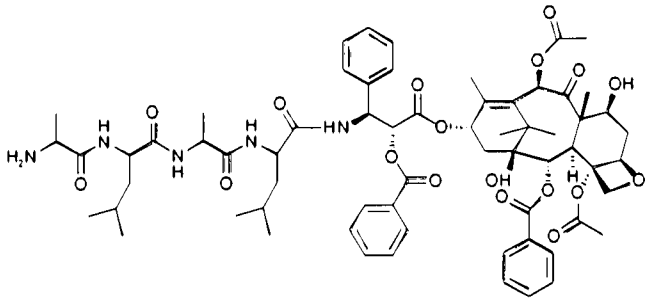


Isotaxel (40 mg, 0.047 mmol, 1 eq) was dissolved in dimethylformamide (1 mL) at +4°C. *N,N*-diisoproylethylamine (28 µL, 0.16 mmol, 3.5 eq) was added, followed by a solution of Fmoc-ALAL-OH (92 mg, 0.14 mmol, 3 eq) in dimethylformamide (0.5 mL). The solution was stirred at +4°C for 10 minutes then *O*-(7-azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (160 mg, 0.70 mmol, 1.5 eq) in dimethylformamide (0.2 mL) was added dropwise. The solution was stirred at +4°C for 10 minutes, then 1 hour at room temperature. Dichloromethane (4 mL) was added and the solution was washed twice with NaCl 1M (2 mL) and twice with aqueous citric acid 5% (2 mL). The organic layer was concentrated under vacuum.

Purity: 90% (determined by HPLC at 214 nm).

MS (ES^+): 1444 $[MH]^+$

I.13.2 Synthesis of ALAL-isotaxel



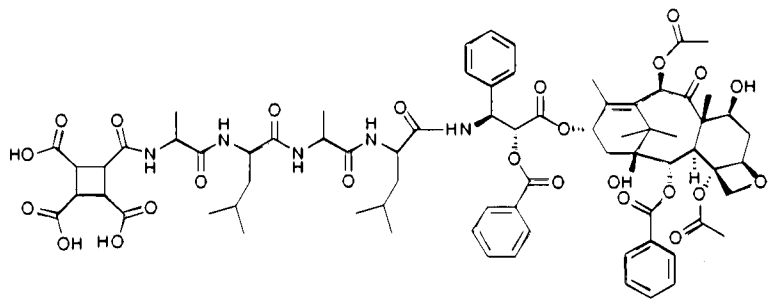
Fmoc-ALAL-isotaxel (50 mg, 0.035 mmol, 1 eq) was dissolved in dimethylformamide (0.74 mL), and piperidine (170 µL, 1.75 mmol, 50 eq) was added. The solution was stirred at room temperature for 5 minutes and directly quenched at +4°C with HCl 6N (0.29 mL, 1.75 mmol, 50 eq) to obtain pH 6.5.

Dichloromethane (4 mL) was added and the solution was washed twice with NaCl 1M (2 mL) and twice with aqueous citric acid 5% (2 mL). The organic layer was dried over Na_2SO_4 and purified by flash chromatography (DCM/MeOH 90:10). The recovered fractions were pooled and concentrated under vacuum to obtain a pale yellow oil.

Purity: 90% (determined by HPLC at 214 nm).

MS (ES^+): 1222 $[MH]^+$

I.13.3 Synthesis of TA-ALAL-isotaxel



ALAL-Isotaxel (20 mg, 0.016 mmol, 1 eq) was dissolved in dimethylformamide (0.5 mL) and *N,N*-diisopropylethylamine (0.015 mL, 0.08 mmol, 5 eq) was added. The solution was stirred 5 minutes at room temperature then 1,2,3,4-cyclobutanetetracarboxylic acid (30 mg, 0.13 mmol, 8 eq) was added followed by *O*-(7-azabenzotriazol-1-yl)-*N,N,N',N'*tetramethyluronium hexafluorophosphate (9 mg, 0.024 mmol, 1.5 eq). The solution was stirred 1 hour at room temperature.

Dichloromethane (5 mL) was added and the solution was washed twice with NaCl 1M (2 mL) and twice with aqueous citric acid 5% (2 mL). The organic layer was dried over

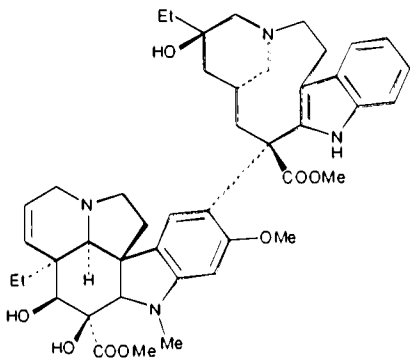
Na_2SO_4 and concentrated under vacuum. Acetonitrile (0.5 mL) and water (0.5 mL) were added and the pH of the solution was adjusted at 7 with NaOH 1M. The solution was purified by preparative HPLC. The recovered HPLC fractions were pooled and lyophilized.

Purity: 99.2% (determined by HPLC at 214 nm).

MS (ES^+): 1434 $[MH]^+$

I.14 Method of synthesis of TA-ALAL-deacetylvinblastine

I.14.1 Synthesis of deacetylvinblastine

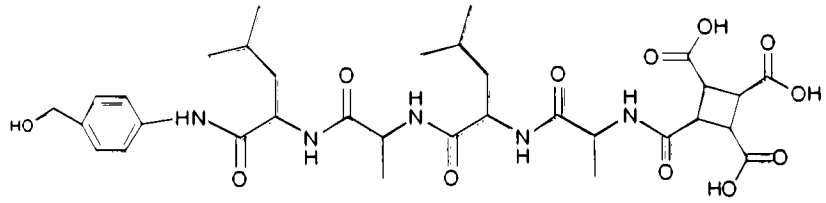


Vinblastine sulfate (3 g, 3.7 mmol, 1 eq) was dissolved in anhydrous methanol (100 mL) and a solution of sodium methoxide 25% in methanol (5.6 mL) was added. The mixture was refluxed under argon during 2 hours. The pH of the reaction was adjusted to 1 by adding HCl 1.25M in methanol. Water (100 mL) was added and the pH was adjusted to 8-8.5 with NaOH 1M. The organic layer was extracted 3 times with dichloromethane. The organic layer was dried over Na_2SO_4 and filtered. The solvent was evaporated under vacuum.

Purity: 99% (determined by HPLC at 214 nm).

MS (ES^+): 770 $[MH]^+$

I.14.2 Synthesis of TA-ALAL-aminobenzylalcohol



Fmoc-ALAL-OH (3 g, 5 mmol, 1 eq) was dissolved in dimethylformamide (20 mL) and 4-aminobenzylalcohol (0.728 g, 6 mmol, 1.2 eq) and 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (2.45 g, 6 mmol, 1.2 eq) were added. The pH of the reaction was adjusted to 8-8.5 with *N,N*-diisopropylethylamine. The mixture was stirred 30 min. The reaction mixture was precipitated in water and filtered. The product was freeze-dried .

MS (ES^+): 714 $[MH]^+$

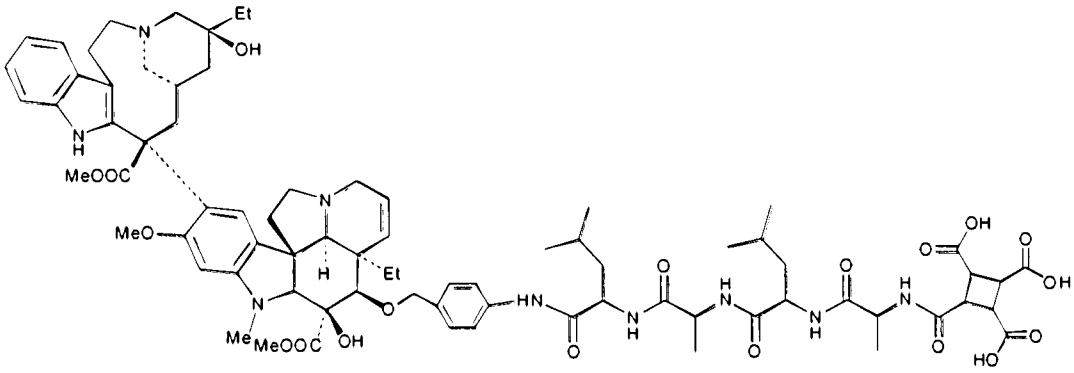
Fmoc-ALAL-aminobenzylalcohol (3.570 g, 5 mmol, 1 eq) was dissolved in dimethylformamide (20 mL) and piperidine (20% of final volume) was added. The mixture was stirred for 5 minutes at room temperature. The reaction was precipitated in water and filtered to obtain NH_2-ALAL-aminobenzylalcohol. The filtrate was loaded on YMC®Gel ODS-A (C18 phase, 12 nm, s-50 µm). The product was eluted in methanol and the solvent was evaporated. The product was freeze-dried.

MS (ES^+): 492 $[MH]^+$

NH_2-ALAL-aminobenzylalcohol (2.46 g, 5 mmol, 1 eq) was dissolved in dimethylformamide (20 mL) and 1,2,3,4-cyclobutanetetracarboxylic acid (11.6 g, 50 mmol, 10 eq) was added. The pH of the reaction was adjusted to 7.5-8 with *N,N*-diisopropylethylamine. 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (2.45 g, 6 mmol, 1.2 eq) was added to the mixture and the pH was verified. The reaction was diluted in water (40 mL) and loaded on YMC®Gel ODS-A (C18 phase, 12 nm, s-50 µm). The product was eluted in methanol and the solvent was evaporated. The product was freeze-dried. TA-ALAL-aminobenzylalcohol was purified by preparative HPLC.

MS (ES^-): 704 $[MH]^-$

I.14.3 Synthesis of TA-ALAL-deacetylvinblastine

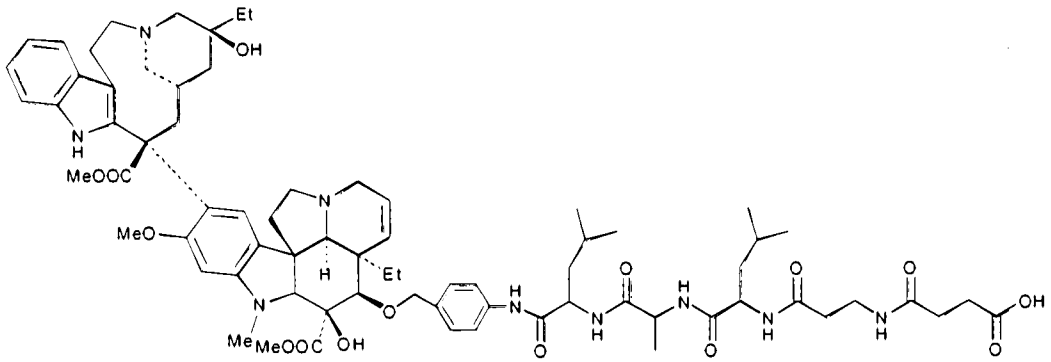


TA-ALAL-aminobenzylalcohol (50 mg, 0.071 mmol, 2 eq) was dissolved in acetonitrile (5 mL) and di-(*N*-succinimidyl)carbonate (90 mg, 0.355 mmol, 10 eq) was added. The pH of the reaction was adjusted to 9-9.5 with *N,N*-diisopropylethylamine. The reaction was stirred for 4 hours at room temperature and the solvent was evaporated. The mixture was dissolved in chloroform (20 mL) and filtered. After evaporation of chloroform under vacuum, the activated TA-ALAL-aminobenzylalcohol was resuspended in dimethylformamide (5 mL) and added to a solution of deacetylvinblastine (54.5 mg, 0.36 mmol, 1 eq) in dimethylformamide (5 mL). The mixture was heated at 50°C and stirred for 4 hours. The mixture was then diluted in water (50 mL) and loaded on YMC®Gel ODS-A (C18 phase, 12 nm, s-50 μm). The product was eluted in methanol and the solvent was evaporated. The TA-ALAL-ether-deacetylvinblastine (further called TA-ALAL-deacetylvinblastine) was purified by preparative HPLC.

Purity: 90-95% (determined by HPLC at 214 nm).

MS (ES^+): 1456.5 $[MH]^+$

I.15 Synthesis of succinyl-βALAL-deacetylvinblastine



OMe-succinyl-βALAL-OH (20 mg, 0.033 mmol, 1 eq) was dissolved in dimethylformamide (5 mL) and 4-aminobenzylalcohol (4.9 mg, 0.04 mmol, 1.2 eq) was

added. The pH of the reaction was adjusted to 8 with *N,N*-diisopropylethylamine and 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (16.5 mg, 0.04 mmol, 1.2 eq) was added. The reaction was stirred 1 hour at room temperature, diluted in water (10 mL) and the solution was loaded on YMC®Gel ODS-A (C18 phase, 12 nm, s-50 µm). The product was eluted with methanol and the solvent was evaporated. OMe-succinyl-βALAL-aminobenzylalcohol (20 mg, 0.033 mmol, 1 eq) was dissolved in acetonitrile (5 mL) and di-(*N*-succinimidyl)carbonate (10.1 mg, 0.04 mmol, 1.2 eq) was added. The pH of the reaction was adjusted to 9-9.5 with *N,N*-diisopropylethylamine. The mixture was stirred for 2 hours and the solvent was evaporated. The mixture was then dissolved in chloroform (5 mL), filtered and evaporated. The product was dissolved in dimethylformamide (5 mL) and added to a solution of deacetylvinblastine (5 mg, 0.0066 mmol, 0.2 eq) in dimethylformamide (2 mL). The mixture was incubated at 50°C for 4 hours.

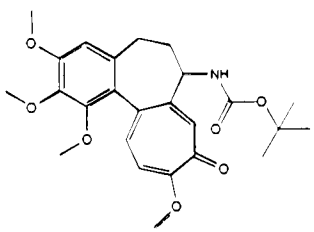
The mixture was then precipitated in diethylether (50 mL) and filtered. The precipitate was resuspended in methanol and the solvent was evaporated. The OMe-succinyl-βALAL-ether-deacetylvinblastine was dissolved in dimethylformamide (0.5 mL) and the product was added to a solution of CLEA enzyme (10 mg) in PBS pH 7.4 (2 mL). The mixture was incubated at 37°C overnight. The mixture was then centrifuged. The pellet was diluted in water and loaded on YMC®Gel ODS-A (C18 phase, 12 nm, s-50 µm). The product was eluted with methanol and the solvent was evaporated. The succinyl-βALAL-ether-deacetylvinblastine (further called succinyl-βALAL-deacetylvinblastine) was purified by preparative HPLC.

Purity: 90% (determined by HPLC at 214 nm).

MS (ES^+): 1342 $[MH]^+$

I.16 Method of synthesis of TA-ALAL-deacetylcolchicine

I.16.1 Synthesis of *N*-(*t*-Boc)deacetylcolchicine



Colchicine (1 g, 2.5 mmol, 1 eq) was dissolved in dichloromethane (10 mL) and 4-dimethylaminopyridine (0.340 g, 2.8 mmol, 1.1 eq) followed by triethylamine (0.4 mL, 2.8 mmol, 1.1 eq). Di(*tert*-butyl)dicarbonate (3 g, 14 mmol, 5.6 eq) was added by portions to the mixture and stirred overnight at room temperature. The reaction mixture was precipitated in diethylether and filtered. The filtrate was evaporated and the residue was redissolved in dimethyformamide (4 mL) and water (50 mL). The product was loaded on YMC®Gel ODS-A (C18 phase, 12 nm, s-50 µm) and washed with an aqueous solution of methanol (gradient 5% to 30 %). The *N*-(*t*-Boc)colchicine was eluted with methanol and the solvent was removed by rotary evaporation.

Purity: 90-95 % determined by HPLC at 254 nm and 350 nm.

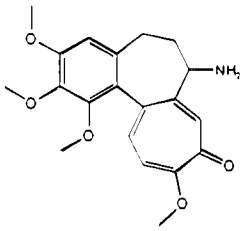
MS (ES^+): 500.2 $[MH]^+$

N-(*t*-Boc)colchicine (0.625 g, 1.25 mmol, 1 eq) was dissolved in methanol (5 mL) and a solution of sodium methoxide 25% in methanol (0.08 mL) was added. The mixture was stirred for 2 hours at 0°C. The reaction was neutralized by adding solid NH_4Cl. The product was diluted in water (20 mL) and loaded on YMC®Gel ODS-A (C18 phase, 12 nm, s-50 µm) and washed with an aqueous solution of methanol 20%. The *N*-(*t*-Boc)deacetylcolchicine was eluted with methanol and the solvent was removed by rotary evaporation.

Purity: 90-95% (determined by HPLC at 254 nm and 350 nm.)

MS (ES^+): 458.1 $[MH]^+$

I.16.2 Synthesis of deacetylcolchicine



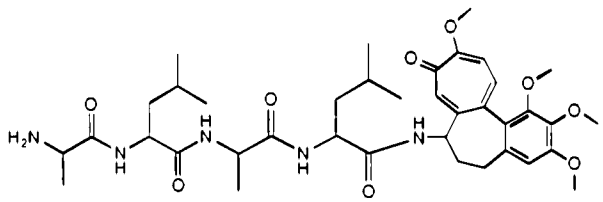
N-(*t*-Boc)deacetylcolchicine (0.572 g, 1.25 mmol, 1 eq) was dissolved in dichloromethane (5 mL) and trifluoroacetic acid (1 mL) was added. The mixture was

stirred at room temperature for 30 min. The reaction was precipitated in diethylether and filtered. The precipitate was resuspended in methanol (10 mL) and the solvent was evaporated. Deacetylcolchicine was purified by preparative HPLC.

Purity: 99 % determined by HPLC at 254 nm and 350 nm.

MS (ES^+): 358.2 $[MH]^+$

I.16.3 Synthesis of NH_2-ALAL-deacetylcolchicine

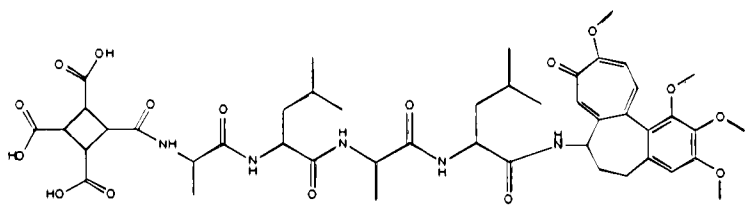


Deacetylcolchicine (75 mg, 0.21 mmol, 1 eq) and Fmoc-ALAL-OH (0.153 g, 0.252 mmol, 1.2 eq) were dissolved in dimethylformamide (4 mL). The pH was adjusted to 7,5 with *N,N*-diisopropylethylamine and 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (0.104 g, 0.252 mmol, 1.2 eq) was added. After 30 minutes, piperidine (1 mL) was added (20 % final volume) and the mixture was stirred for 5 minutes at room temperature. A 10 % lactate solution in water (pH 3) was added for neutralization. The reaction mixture was loaded on YMC®Gel ODS-A (C18 phase, 12 nm, s-50 µm) and washed with an aqueous solution of methanol (gradient 25% to 35%) The product was eluted in methanol and the solvent was evaporated.

Purity: 50% determined by HPLC (214 nm)

MS (ES^+): 726 $[MH]^+$

I.16.4 Synthesis of TA-ALAL-deacetylcolchicine

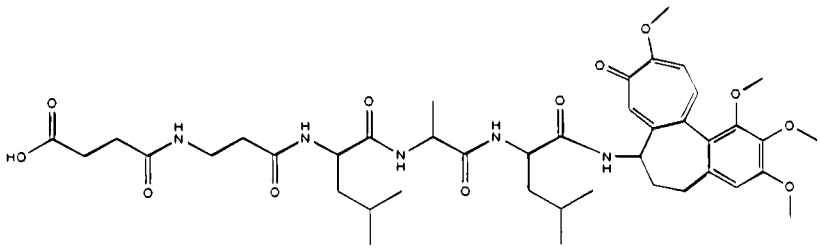


NH_2-ALAL-deacetylcolchicine (0.152 g, 0.21 mmol, 1 eq) and 1,2,3,4-cyclobutane tetracarboxylic acid (0.487 g, 2.1 mmol, 10 eq) were dissolved in dimethylformamide (5 mL). The pH was adjusted to 7.5 with *N,N*-diisopropylethylamine and 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (0.104 g, 0.252 mmol, 1.2 eq) was added. The solution was stirred at room temperature for 45 minutes and the mixture was precipitated in diethylether and filtered. The precipitate was dissolved in methanol (20 mL) and the solvent was evaporatd. The product was purified by preparative HPLC.

Purity: 99% (determined by HPLC at 254 nm and 350 nm)

MS (ES^-): 938 $[MH]^-$

I.17 Synthesis of succinyl-βALAL-deacetylcolchicine



Deacetylcolchicine (75 mg, 0.21 mmol, 1 eq) and OMe-succinyl-βALAL-OH (0.153 g, 0.252 mmol, 1.2 eq) were dissolved in dimethylformamide (3 mL). The pH was adjusted to 7.5 with *N,N*-diisopropylethylamine and 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (0.104 g, 0252 mmol, 1.2 eq) was added. The reaction was stirred 30 minutes and the mixture was precipitated in diethylether (100 mL) and filtered. The precipitate was resuspended in methanol (20 mL) and the solvent was evaporated. The OMe-succinyl-βALAL-deacetylcolchicine was dissolved in dimethylformamide and pH was adjusted to 14 by adding NaOH 1M. After stirring 1 hour, the product was precipitated in diethylether and filtered. The precipitate was resuspended in methanol and the solvent was evaporated. The succinyl-βALAL-deacetylcolchicine was dissolved in water (100 mL) and lyophilized.

Purity: 90% (determined by HPLC at 254 nm and 350 nm)

MS (ES^+): 826 $[MH]^+$

II. *In vitro* human plasma stability studies of TA-ALAL-drug conjugates

II.1 Method

Human plasma (citrated human blood was centrifuged at 600 *g* for 10 min at +4°C, plasma was recovered and aliquots stored at -20°C) was used to assess the stability of the following drug conjugates: succinyl-ALAL-doxorubicin, succinyl-βALAL-doxorubicin, TA-ALAL-doxorubicin, succinyl-βALAL-mitomycin C, TA-ALAL-mitomycin C, succinyl-βALAL-paclitaxel and TA-ALAL-paclitaxel, TA-ALAL-docetaxel, TA-ALAL-isotaxel, succinyl-βALAL-deacetylcolchicine, TA-ALAL-deacetylcolchicine, succinyl-βALAL-deacetylvinblastine, TA-ALAL-deacetylvinblastine, TA-ALAL-geldanamycin and TA-ALAL-ispinesib.

The drug conjugates (50 µM doxorubicin, deacetylvinblastine or mitomycin C, 100 µM paclitaxel or deacetylcolchicine and 300 µM for other conjugates) were incubated at 37°C with human plasma for 5-6 hours, 50 µL samples were collected at different time points and extraction was performed immediately.

For the doxorubicin, deacetylvinblastine, ispinesib and geldanamycin conjugates and metabolites, 150 µL of acetonitrile were added to the 50 µL samples. Samples were vortexed and centrifuged for 10 min at 13 000 rpm at room temperature. 100 µL of supernatant were collected and 300 µL of 200 mM formate buffer pH 4.5 were added before centrifugation (10 min at 13 000 rpm). HPLC analysis was performed using the supernatant at the indicated wavelength: 254 nm for doxorubicin and geldanamycin, 280 nm for vinblastine and 236 nm for ispinesib derivatives.

For mitomycin C conjugate and metabolites, 100 µL of acetonitrile was added to the 50 µL sample, prior to vortexing (10 seconds) and centrifugation (10 min at 13 000 rpm) at room temperature. 50 µL of supernatant was recovered and mixed with 150 µL of phosphate buffered saline (PBS). After centrifugation (10 min at 13 000 rpm), 150 µL of supernatant were collected for HPLC analysis. Stability and reactivation were evaluated by HPLC analysis (at 356 nm) and mitomycin C conjugate and metabolite peak areas were compared.

For paclitaxel, docetaxel and isotaxel conjugates and metabolites, 150 µL of acetonitrile was added to 50 µL of sample, vortexed and centrifuged for 10 min at 13 000 rpm at room temperature. 100 µL of supernatant was recovered and mixed with 100 µL of water.

After centrifugation (10 min at 13 000 rpm), the supernatant was collected for HPLC analysis (at 254 nm).

For deacetylcolchicine conjugates and metabolites, the 50 μL samples (+ 10 μL of 500 μM colchicine, internal standard) were precipitated with 150 μL of acetonitrile. The precipitate was eliminated by centrifugation, 150 μL of supernatant was collected and evaporated under an air flow. The residue was dissolved in 200 μL water or 2% formate buffer pH 4, homogenized by sonification and analyzed by HPLC (at 350 nm).

II.2 Results

All three doxorubicin conjugates succinyl-ALAL-doxorubicin, succinyl-βALAL-doxorubicin and TA-ALAL-doxorubicin were stable in human plasma after 2 hours of incubation and improved stability could be detected for the conjugate with TA: the percentage of intact conjugate observed after 2 hours in human plasma was 80, 77.2 and 83% for succinyl-ALAL-doxorubicin, succinyl-βALAL-doxorubicin and TA-ALAL-doxorubicin, whilst after 6 hours the percentage of intact conjugate was 54, 47.5 and 67% respectively.

The TA-ALAL-mitomycin C conjugate was slightly more stable than the succinyl-βALAL-mitomycin C conjugate, with respectively 78% and 73% of conjugates remaining after 6 hours. The same observation was made for succinyl-βALAL-geldanamycin and TA-ALAL-geldanamycin with high human plasma stability (75% and 88% of conjugates, respectively) after 6 hours.

The plasma stability of succinyl-βALAL-paclitaxel and TA-ALAL-paclitaxel conjugates was also similar (41 and 43% respectively after 6 hours).

Docetaxel, isotaxel, ispinesib, deacetylvinblastine and deacetylcolchicine conjugates were stable in human plasma as no degradation or significant release of free drug or metabolites was detected after 5-6 hours incubation at 37°C.

These studies show that the TA moiety improves the in vitro stability of the conjugate.

III *In vitro* evaluation of cytotoxic activity of intact TA-ALAL-drug conjugates

III.1 Method

The cytotoxicity of different prodrug conjugates was evaluated using the HCT 116 colon cancer cell line and was compared to the cytotoxicity of the free drug (doxorubicin,

mitomycin C, paclitaxel, docetaxel, ispinesib, deacetylvinblastine, deacetylcolchicine or 17DMAG). Cells were seeded at between 8 000-10,000 cells/well and maintained in cell culture medium supplemented with 10% fetal bovine serum for 24 hours before treatment. Cells were incubated with increasing concentrations of drugs or conjugates at 37°C, 5% CO_2. The medium was then removed and a WST-1 test (purchased from Roche Applied Science) was performed according to the manufacturer's instructions.

The cytotoxicity of the tested conjugates was expressed as the molar concentration inhibiting 50% of the cell viability (IC_{50}), calculated using Graph Pad Prism 3.02 software. Experimental data are presented as the mean of three independent experiments performed in duplicate.

III.2 Results

The intact prodrug should be inactive or significantly less active in comparison with the free drug and should only be reactivated by tumor selective endopeptidases in the tumor environment. *In vitro* experiments were performed in the HCT 116 cell line to confirm that the prodrug conjugate is inactive compared to its corresponding free drug. HCT 116 cells do not secrete sufficient endopeptidases into the culture medium to allow activation of the prodrug, under the conditions of the test. The cytotoxicity of the different prodrug conjugates is shown in Table 1 below. All drug conjugates (TA-ALAL-drug, succinyl-ALAL-drug or succinyl-βALAL-drug) showed a significant level of inactivation compared to the non-conjugated drug. This data confirms that conjugation of TA-ALAL peptide to the cytotoxic drugs tested results in inactivation of the cytotoxic drug, presumably through inhibition of the internalisation of the cytotoxic molecule thus preventing its interaction with its therapeutic target. Comparing the TA and succinyl-β stabilizing groups, the TA-ALAL prodrugs show equivalent or improved inactivation characteristics compared to succinyl-βALAL or succinyl-ALAL prodrugs.

Table 1: Drug concentration that inhibits 50% of cell viability (IC_{50}) using the HCT 116 cell line. Data represent the mean value of 3 independent experiments. After the indicated exposure time at 37°C, cells were rinsed and a viability assay (WST1 test) was performed to determine IC_{50}.

Drugs or drug conjugates	IC50 values (μM)	Incubation time
doxorubicin (dox)	0.034	48 h
TA-ALAL-dox	> 10	48 h
Suc-βALAL-dox	> 10	48 h
Suc-ALAL-dox	10	48 h
mitomycin C (MMC)	5	24 h
TA-ALAL-MMC	86	24 h
Suc-βALAL-MMC	67	24 h
paclitaxel	0.020	4h + 72 h
TA-ALAL-paclitaxel	10	4h + 72 h
Suc-βALAL-paclitaxel	0.762	4h + 72 h
docetaxel	0.0025	4h + 72 h
TA-ALAL-docetaxel	0.410	4h + 72 h
ispinesib	0.0025	48 h
TA-ALAL-ispinesib	0.410	48 h
17-dimethylamino-geldanamycin	0.034	48h
Suc-βALAL-geldanamycin	> 50	48h
TA-ALAL-geldanamycin*	> 50	48h
deacetylvinblastine (deaVLB)	0.003	48 h
TA-ALAL-deaVLB	0.326	48 h
Suc-βALAL-deaVLB	0.646	48 h
deacetylcolchicine	0.04	48 h
TA-ALAL-deacetylcolchicine	91	48 h
Suc-βALAL-deacetylcolchicine	1.5	48 h

*Data represent the mean value of duplicate samples from one experiment.
Suc = succinyl

IV Prodrug reactivation and drug release

IV.1 Method

LS 174T tumors were collected from xenografted mice 17 days after implantation (tumor weight between 400 and 600 mg) and stored at -80°C. To obtain a homogenate, the tumor was thawed and transferred into a 10 mL glass tube containing water (1 mL water/200 mg material). The tumor was homogenized using an Ultra-Turax homogenizer followed by sonification at 4°C. The homogenate was then buffered with Tris-HCl pH 7.4 (1V 1M Tris-HCl + 49 V LS 174T tumor homogenate) and stored at -20°C until use.

TA-ALAL-drugs were incubated in LS 174T tumor homogenate at a concentration described in the *in vitro* human plasma stability method section. Fifty microliters were collected at 2 hours and 24 hours of incubation and processed as previously described for HPLC analysis.

IV.2 Results

Transformation of the prodrugs into the active drug was measured in the presence of peptidases from LS 174T tumor homogenates using HPLC. Under these conditions, cleavage of all the prodrugs listed was observed with simultaneous apparition of the Leu-drug metabolite and/or the free drug. Prodrugs containing the TA moiety can therefore be enzymatically reactivated (Table 2).

Table 2: Summary of TA-ALAL-drug reactivation in the presence of peptidases from LS 174T tumor homogenates; nd, not determined

Compounds	Metabolite release (% of total substrate) after 2h	after 24h
TA-ALAL-dox	8%	10%
TA-ALAL-MMC	3%	25%
TA-ALAL-paclitaxel	13%	45%
TA-ALAL-ispinesib	nd	14%
TA-ALAL-geldanamycin	44%	nd
TA-ALAL-deacetylcolchicine	nd	90%

V Evaluation of the *in vivo* toxicity of TA-ALAL-doxorubicin conjugate compared to known doxorubicin conjugates

Toxicity studies were conducted in LS 174T tumor-bearing nude mice following intravenous administration. This model was used to assess the toxicity of TA-ALAL-doxorubicin as this model has been shown to over-express CD10 as well as TOP. This

model has also been shown to be responsive to succinyl-βALAL-doxorubicin (Dubois *et al.*, Cancer Research, 62(8), 2327-2331 (2002)) [4].

LS 174T tumors were established by a subcutaneous implantation of cells (3×10^7 cells injected) in the right flank of 7 weeks old female NMRI nude mice (Janvier, France). The day of the first injection, animals were randomly assigned to groups of 6 animals. All conjugates were administered by bolus intravenous injection (i.v.) in the lateral tail vein at 10 µL/g. During the course of the experiment, clinical signs and body weight were controlled at least twice a week. The experiment was repeated twice. Results are shown in Table ~~Table~~ Table 3 below.

Table 3: *In vivo* toxicity of TA-ALAL-doxorubicin conjugate compared to succinyl-βALAL-doxorubicin and succinyl-ALAL-doxorubicin in LS 174T tumor-bearing mice.

	Doxorubicin conjugates	Dose (µmol/kg)	Maximal body weight loss %
Experiment 1	TA-ALAL-dox	67.7	13.7
	Suc-βALAL-dox	67.7	19.9
	Suc-ALAL-dox	67.7	36.1 (3 deaths)
Experiment 2	TA-ALAL-dox	55.1	11.3
		77.4	17.9
	Suc-βALAL-dox	33.8	10.6
		47.4	15.9

At equivalent doses (experiment 1, Table 3) TA-ALAL-dox induced less body weight loss than succinyl-βALAL-dox, suggesting that TA-ALAL-dox has a better blood stability than succinyl-βALAL-dox *in vivo*. On the contrary, replacement of the βAla by an Ala in the succinyl conjugate induced a loss of *in vivo* stability as an equivalent dose of succinyl-ALAL-dox produced an important body weight loss and the death of 3 animals in the group. Experiment 2 (Table 3) shows the doses of TA-ALAL-dox and succinyl-βALAL-dox required to have an equitoxic effect. This experiment is consistent with experiment 1, confirming that TA-ALAL-dox is less toxic than succinyl-βALAL-dox and shows that equitoxic doses of TA-ALAL-dox and succinyl-βALAL-dox are 77.4 and 47.4 µmol/kg respectively.

VI Evaluation of the *in vivo* efficacy of TA-ALAL-doxorubicin conjugate compared to known doxorubicin conjugates in the LS 174T tumor model

LS 174T tumors were established by a subcutaneous implantation of cells (3×10^7 cells injected) in the right flank of 7 week old female NMRI nude mice (Janvier, France). Treatments were initiated when the tumors had reached a size of 100 mm^3 (calculated using the following formula: [length x width^2]/2). The day of the first injection animals were randomly assigned to groups of 6 animals. The conjugates were administered by bolus intravenous injection (i.v.) in the lateral tail vein at 10 µL/g. During the course of the experiment, clinical signs, body weight and tumor volume were controlled twice a week. Results are presented as the evolution of mean tumor volume as a function of time. Optimal T/C (ratio of mean tumor volume of treated *versus* control mice) values were used as a measure of treatment efficacy. The lower the T/C%, the greater the efficacy of the product. The experiment was repeated twice. Results are shown in Table 4 below.

Table 4: Anti-tumoral efficacy of TA-ALAL-doxorubicin compared to succinyl-βALAL-doxorubicin and succinyl-ALAL-doxorubicin in the LS 174T tumor model.

	Doxorubicin conjugates	Dose (µmol/kg)	Optimal T/C (%)	Maximal body weight loss %
Experiment 1	TA-ALAL-dox	67.7	42	13.7
	Suc-βALAL-dox	67.7	52	19.9
	Suc-ALAL-dox	24.2*	70	7.3
	Suc-ALAL-dox	67.7	3 animals died	34.4
Experiment 2	TA-ALAL-dox	77.4	36	13.7
	Suc-βALAL-dox	47.4	52	13.5

* Higher dose was toxic

In these experiments, TA-ALAL-dox shows an increased antitumoral efficacy as compared to both succinyl-βALAL-dox and succinyl-ALAL-dox.

VII Evaluation of the *in vivo* efficacy of TA-ALAL-mitomycin C conjugate in the LS 174T tumor model

The *in vivo* anti-tumoral efficacy of TA-ALAL-MMC was compared to that of succinyl-βALAL-MMC in the LS 174T tumor model.

The establishment of the LS 174T tumors and the treatments were carried out as previously described in Example VI. The conjugates were administered by bolus intravenous injection (i.v.) in the lateral tail vein at 10 µL/g. During the course of the experiment, clinical signs, body weight and tumor volume were controlled twice a week. The experiment was repeated twice. Results are shown in Table Table Table 5 below.

Table 5: Anti-tumoral efficacy of TA-ALAL-MMC compared to succinyl-βALAL-MMC in the LS 174T tumor model.

Conjugates	Dose (µmol/kg)	Optimal T/C (%)	Maximal body weight loss %
Experiment 1			
TA-ALAL-MMC	60	28	16.2
Suc-βALAL-MMC	60	48	14.5
Experiment 2			
TA-ALAL-MMC	60	19	19
Suc-βALAL-MMC	80	43	15.9
Suc-ALAL-MMC	40	52	13.7

TA-ALAL-MMC showed an increased antitumoral efficacy as compared to succinyl-βALAL-MMC.

VIII Evaluation of the *in vivo* efficacy of TA-ALAL-ispinesib conjugate

The *in vivo* anti-tumoral efficacy of TA-ALAL-ispinesib was evaluated and compared to that of ispinesib in HT-29 and LS174T tumor models.

The establishment of the LS 174T tumors and the treatments were carried out as previously described in Example VI. HT-29 tumors were established by an intradermal

implantation of cells (10^7 cells injected) in the right flank of 7 weeks old female NMRI nude mice (Janvier, France). Treatments were initiated when the tumors had reached a size of 100 mm^3 (calculated using the following formula: [length x $width^2$]/2). The day of the first injection animals were randomly assigned to groups of 6 animals. The compounds were administered by bolus intravenous injection (i.v.) in the lateral tail vein at 10 µL/g. During the course of the experiment, clinical signs, body weight and tumor volume were controlled twice a week. Results are shown in Table 6 below.

Table 6: Anti-tumoral efficacy of TA-ALAL-ispinesib compared to ispinesib.

Conjugates	Model	Dose (µmol/kg)	Optimal T/C (%)	Maximal body weight loss %
Experiment 1	HT-29			
TA-ALAL-ispinesib		16.3	59	1.4
TA-ALAL-ispinesib		48.9	33	11.1
TA-ALAL-ispinesib		81.6	4	12.9
ispinesib		4.9	62	2.7
ispinesib		9.8	43	9.4
ispinesib*		16.3	28	10.5
Experiment 2	LS 174T			
TA-ALAL-ispinesib		16.3	48	9.4
TA-ALAL-ispinesib		32.6	35	12.9
TA-ALAL-ispinesib		48.9	18	12.9

*ispinesib doses > 9.8 µmol/kg were toxic

TA-ALAL-ispinesib showed a dose dependent antitumoral activity in both models tested. TA-ALAL-ispinesib showed an increased antitumoral efficacy as compared to ispinesib.

IX Evaluation of TA-peptide-drugs solubility

IX.1 Evaluation of TA-peptide-paclitaxel solubility

IX.1.1 Methods

TA-peptide-paclitaxel conjugates including different peptidic moieties (ALAL and KALAL) were tested for their solubility in PBS in comparison with succinyl-ALAL-paclitaxel and paclitaxel.

Ten mg of each compound (TA-ALAL-paclitaxel, succinyl-βALAL-paclitaxel, TA-KALAL-paclitaxel, paclitaxel) were suspended in PBS (0.1 mL). Suspensions were vortexed for 3 minutes and centrifuged for 2 minutes at 13 000 rpm at room temperature. The supernatant was then analysed by HPLC. The concentration of each saturated solution (supernatant) was determined by HPLC by comparison with a paclitaxel standard curve at 270 nm.

IX.1.2 Results

Results are shown in Table 7. TA improves solubility of paclitaxel and TA-ALAL-paclitaxel is more (300 times) soluble than succinyl-βALAL-paclitaxel. The addition of a lysine residue as in TA-KALAL-paclitaxel increased the solubility even further.

Table 7: Solubility of X-peptide-paclitaxel in PBS.

paclitaxel conjugates	Saturated concentration in PBS (mg/mL eq paclitaxel)
TA-ALAL-paclitaxel	9.9
succinyl-βALAL-paclitaxel	0.03
TA-KALAL-paclitaxel	30
paclitaxel	0.53×10^{-3}

IX.2 Evaluation of TA-ALAL-mitomycin C solubility

IX.2.1 Methods

TA-ALAL-mitomycin C conjugate and mitomycin C were tested for their solubility in PBS. Twenty mg of each compound (TA-ALAL-mitomycin C, mitomycin C) were solubilized in PBS (0.05 mL). Suspensions were vortexed for 3 minutes and centrifuged

for 2 minutes at 13 000 rpm at room temperature. The supernatant was then analysed by HPLC. The concentration of each saturated solution (supernatant) was determined by HPLC by comparison with a mitomycin C standard curve at 352 nm.

IX.2.2 Results

Results are shown in Table 8. TA significantly improves the solubility of mitomycin C.

Table 8: Solubility of TA-ALAL-mitomycin C compared to mitomycin C in PBS.

mitomycin C compounds	Saturated concentration in PBS (mg/mL eq MMC)
TA-ALAL-MMC	75.5
mitomycin C	1.12

X *In vitro* human plasma stability studies of TA-peptide-doxorubicin conjugates (HPLC analysis)

X.1 Method

TA-peptide-drug conjugates including different peptidic moieties (AANL, a legumain substrate; ALKLL, a plasmin substrate; HSSKLQL, a PSA substrate; and AYGGFL) were tested for their stability in human plasma in comparison with TA-ALAL-doxorubicin. Succinyl-βALAL-doxorubicin was tested in parallel.

The drug conjugates (50 µM) were incubated at 37°C with human plasma (citrated human blood was centrifuged at 600 *g* for 10 min at +4°C, plasma was recovered and aliquots stored at -20°C) either for 0, 1, 3 or 5 hours. 50 µL samples were collected at each time point and an extraction was performed immediatelyby adding 150 µL of acetonitrile to the 50 µL samples. A 50 µM daunorubicin solution (10 µl) was added as internal standard and used for calculating concentrations. Samples were vortexed and centrifuged for 10 min at 13 000 rpm at room temperature. One hundred µL were collected and 300 µL of 2% ammonium formate buffer pH 4 were added before centrifugation (3 min at 13 000 rpm). HPLC analysis was performed using the supernatant.

X.2 Results

All TA-peptide-doxorubicin conjugates and succinyl-βALAL-doxorubicin show good plasma stability. Results of HPLC analysis show that for all tested compounds, hydrolysis into active metabolites (doxorubicin + leucyl-doxorubicin) is low, with a maximum of 8% for TA-HSSKLQL-doxorubicin after 5 hours incubation in human plasma (Table 9). Other metabolites were also detected and the total percentage of metabolites released after 5 hours was below 20% except for TA-ALKLL-doxorubicin, which was the least stable compound.

Table 9. Plasma stability of TA-peptide-doxorubicin conjugates

Compounds	% metabolites released after 5 hours incubation in human plasma		
	doxorubicin	leucyl-doxorubicin	total metabolites
TA-AANL-doxorubicin	0	7	19
TA-ALKLL-doxorubicin	4	3	46
TA-ALAL-doxorubicin	0	3	12
TA-AYGGFL-doxorubicin	1	0	17
TA-HSSKLQL-doxorubicin	0	8	11
Suc-βALAL-doxorubicin	3	4	17

XI *In vitro* reactivation of TA-peptide-doxorubicin conjugates by tumor peptidases (HPLC analysis)

XI.1 Method

TA-AANL-doxorubicin, TA-ALKLL-doxorubicin, TA-ALAL-doxorubicin, TA-HSSKLQL-doxorubicin, TA-AYGGFL-doxorubicin and succinyl-βALAL-doxorubicin conjugates (50 µM) were incubated for 0, 1, 3 or 5 hours in the presence of LS 174T tumor homogenate (prepared as detailed above). Fifty µL samples were collected at each time point and processed as described above.

XI.2 Results

Table 10 shows the results of hydrolysis assays after a 5-hour incubation. All the TA peptide prodrugs are cleaved by tumor peptidases to release leucyl-doxorubicin (L-dox) and doxorubicin (dox) metabolites.

Table 10. *In vitro* reactivation of TA-peptide-doxorubicin into metabolites by tumor homogenates

Compounds	% metabolites released after 5 hours incubation in LS174T tumor homogenate		
	dox	L-dox	total
TA-AANL-doxorubicin	10	90	100
TA-ALKLL-doxorubicin	11	87	98
TA-ALAL-doxorubicin	12	88	100
TA-AYGGFL-doxorubicin	6	58	63
TA-HSSKLQL-doxorubicin	5	46	51
suc-βALAL-doxorubicin	13	87	100

XII *In vitro* reactivation of TA-peptide-doxorubicin conjugates by tumor cell secreted peptidases (HPLC analysis)

XII.1 Method

Sub-confluent cultures of LS 174T tumor cells were washed twice with a phosphate buffered saline solution, and fresh culture medium (DMEM-F12 without phenol red) containing 0.02% bovine serum albumin was added (100 µL/cm^2). After a 24 hour incubation, the conditioned medium was collected, centrifuged for 10 minutes at 300 g, buffered with 1 M Tris-HCl, pH 7.4 (1 volume of buffer + 19 volumes of medium) and concentrated 20 times by ultrafiltration (molecular weight cutoff 10 kDa).

TA-AANL-doxorubicin, TA-ALKLL-doxorubicin, TA-ALAL-doxorubicin, TA-HSSKLQL-doxorubicin, TA-AYGGFL-doxorubicin conjugates and succinyl-βALAL-doxorubicin conjugates (50 µM) were incubated for 0, 1, 3 or 5 hours in the presence of LS 174T tumor homogenate (prepared as detailed above). Fifty µL samples were collected at each time point and processed as described above.

XII.2 Results

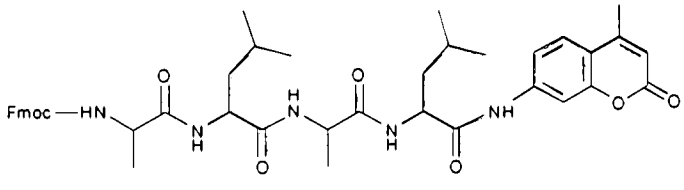
After a 5 hour incubation in the presence of tumor cell conditioned medium, all the TA peptide prodrugs, except for TA-AYGGFL were hydrolyzed into leucyl-doxorubicin (L-Dox) and doxorubicin (Table 11).

Table 11. *In vitro* reactivation of TA-peptide-doxorubicin into metabolites by tumor cell conditioned medium

Compounds	% metabolites released after 5 hours of incubation in LS 174T tumor cell conditioned medium		
	dox	L-dox	total
TA-AANL-doxorubicin	3	34	37
TA-ALKLL-doxorubicin	2	27	29
TA-ALAL-doxorubicin	3	49	52
TA-HSSKLQL-doxorubicin	0	16	16
suc-βALAL-doxorubicin	3	46	49

XIII Method of synthesis of TA-ALAL-Coumarin

XIII.1 Synthesis of Fmoc-ALAL-Coumarin

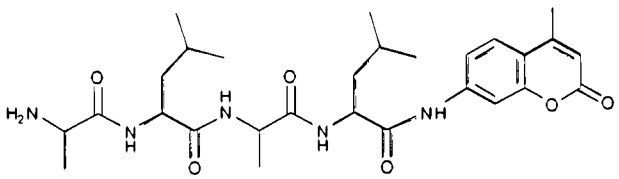


7-Amino-4-methylcoumarin (25 mg, 0.143 mmol, 1 eq) was dissolved in dimethylformamide (1 mL). N,N-diisopropylethylamine (62.3 μL, 0.428 mmol, 3 eq) was added followed by Fmoc-ALAL-OH (130 mg, 0.215 mmol, 1.5 eq). After stirring for 2 minutes, 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (82.6 mg, 0.2 mmol, 1.4 eq) was added. The solution was stirred 1 hour at room temperature. Dichloromethane (2 mL) was added and the organic layer was washed with NaCl 1M (1 mL) and aqueous citric acid 5% (1 mL). The organic layer was dried over $MgSO_4$ and evaporated to obtain an oil.

Purity: 85 % (determined by HPLC at 214 nm).

MS (ES^+): 783 $[MH]^+$

XIII.2 Synthesis of NH_2-ALAL-Coumarin

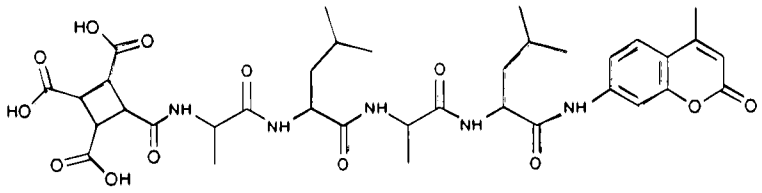


Fmoc-ALAL-Coumarin (30 mg, 0.038 mmol, 1 eq) was dissolved in dimethylformamide (0.5 mL) and piperidine (190 μL, 1.9 mmol, 50 eq) was added. The solution was stirred 3 minutes at room temperature and directly neutralized at +4°C with a cold solution of HCl 6N (317 μL, 1.9 mmol, 50 eq) to obtain a pH of 6. Dichloromethane (2 mL) was added and the organic layer washed with aqueous citric acid 5% (1 mL), NaCl 1M (1 mL). The organic layer was dried over MgSO4, evaporated and the resulting oil purified by flash chromatography (DCM/MeOH 90:10). The recovered fractions were pooled and concentrated by rotary evaporation.

Purity: 90 % (determined by HPLC at 214 nm).

MS (ES^+): 566 $[MNa]^+$

XIII.3 Synthesis of TA-ALAL-Coumarin



NH_2-ALAL-Coumarin (25 mg, 0.046 mmol, 1 eq) was dissolved in dimethylformamide (0.5 mL). N,N-diisopropylethylamine (24.2 μL, 0.138 mmol, 3 eq) was added followed by 1,2,3,4-cyclobutanetetracarboxylic acid (106.7 mg, 0.46 mmol, 10 eq). After stirring 5 minutes, O-(7-Azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluoro phosphate (26.2 mg, 0.069 mmol, 1.5 eq) was added. The solution was stirred 1 hour at room temperature. Dichloromethane (2 mL) was added and the organic layer washed twice with NaCl 1M (3 mL) and twice with aqueous citric acid 5% (3 mL). The organic layer was dried over $MgSO_4$ and evaporated to obtain a solid. The crude solid was suspended in dimethylformamide (1 mL), acetonitrile (1 mL) and water (4 mL) and then purified by preparative HPLC. The recovered HPLC fractions were pooled and lyophilized.

Purity: 95 % (determined by HPLC at 214 nm).
MS (ES$^+$): 756 $[MH]^+$

XIV. TA-peptide-marker compounds for diagnostic purposes

TA-peptide-marker conjugates, such as the TA-ALAL-coumarin synthesised as described above, can be used to diagnose cancerous or other lesions, due to the expression of specific endopeptidases that cleave the TA-peptide marker to liberate the marker molecule, which once free is able to be detected, and thereby to improve cancer diagnosis, improve study of the progression of the tumor, improve assay of the factors secreted by tumor cells, etc.

Conjugation of the marker molecule with a TA-peptide moiety will inhibit the activity of the marker molecule. Enzymatic cleavage of the TA-peptide-marker compound in vitro or in vivo will liberate the free marker molecule and activity of the marker molecule will thus be correlated to enzymatic activity. For example, using TA-ALAL-coumarin, free coumarin can be measured by fluorescence (excitation λ = 350 nm; emission λ = 440

nm) and this assay can be used to measure CD10 and TOP activity, since these enzymes are responsible for cleavage of the ALAL-sequence. The amount of coumarin, expressed as mmol of coumarin liberated/min per mg cell proteins, liberated in homogenates of tumor cells (cancer of the lung, breast, ovaries, etc.) can for example be compared to the amount of coumarin liberated by transformed cells (line of immortalized but noncancerous normal cells) and/or normal cells (fibroblasts and muscle cells).This type of conjugate can also be used *in vivo* to distinguish normal, tumoral or other lesions, as part of a diagnostic kit.

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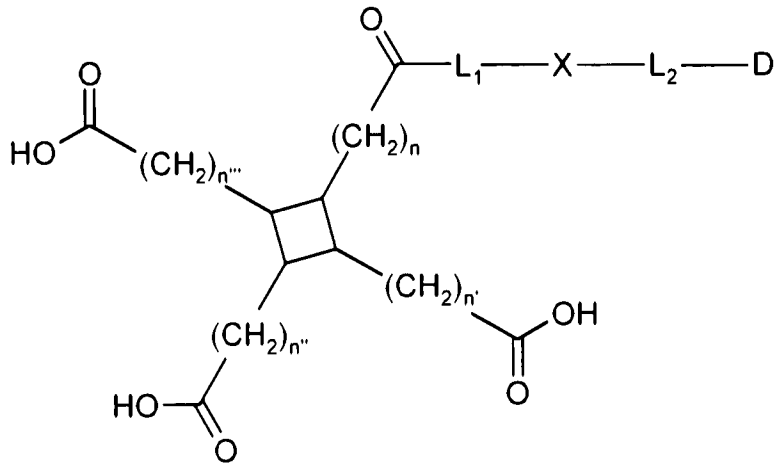
What is claimed is:

1. A compound having the following formula :



(II)

wherein

L_1 and L_2 are independently a covalent bond or a linking moiety, said linking moiety should include at least two organic functional groups, one organic functional group that can bind to the 1,2,3,4-cyclobutanetetracarboxylic acid and a second organic functional group that allows binding to the oligopeptide moiety (L1) or one functional group that can bind to the oligopeptide moiety and a second organic functional group that allows binding to D (L2) ;

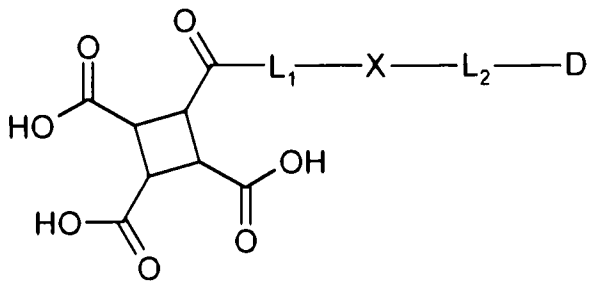
n, n', n'' and n''' independently represent an integer comprised between 0 and 10;

X is a cleavable oligopeptide moiety, said moiety being cleavable by at least one specific and/or selective peptidase that is present in the extracellular environment of target cells;

D is a therapeutic agent or marker ;

or the pharmaceutically acceptable salts thereof.

2. The compound according to claim 1, wherein said compound is represented by formula (III):



(III)

or the pharmaceutically acceptable salts thereof wherein L_1, L_2, D and X are defined as above.

3. The compound according to claim 1 or 2, wherein said cleavable oligopeptide moiety comprises 2 to 10 amino acid residues, preferably 3 to 7 amino acid residues.

4. The compound of claim 1 or 2, wherein the oligopeptide comprises one or more of the following amino acid pairings: Arg-Leu, Arg-Phe, Arg-Val, Ala-Phe, Ala-Ala, Ala-Asn, Ala-Leu, Ala-Tyr, Asn-Leu, Cys-Arg, Cys-Asp, Cys-Phe, Gln-Phe, Gln-leu, Gly-Asp, Gly-Phe, Gly-Leu, Gly-Gln, Gly-Gly, Gly-Pro, His-Ser, Ile-Ala, Leu-Ala, Leu-Gln, Leu-Gly, Leu-Leu, Leu-Phe, Leu-Tyr, Leu, Lys, Lys-Leu, Met-Leu, Pro-Phe, Pro-Tyr, Pro-Leu, Phe-Leu, Phe-Phe, Tyr-Ile, Tyr-Pro, Tyr-Leu, Tyr-Gly, Val-Tyr, Val-Phe, Ser-Ser, Ser-Leu and Ser-Lys.

5. The compound of claim 1 or 2, wherein the oligopeptide comprises one or more of the following sequences: (Leu)y-(Ala-Leu)x-Ala-Leu or (Leu)y-(Ala-Leu)x-Ala-Phe wherein y = 0, 1, 2, 3, 4, or 5 and x = 1, 2, 3, 4 or 5.

6. The compound of claim 1 or 2, wherein the oligopeptide comprises one or more of the following sequences: Ala-Phe-Lys, Ala-Leu-Ala-Leu, Ala-Leu-Lys-Leu-Leu, Ala-Tyr-Gly-Gly-Phe-Leu, His-Ser-Ser-Lys-Leu-Gln-Leu, Gly-Pro-Leu-Gly-Ile-Ala-Gly-Gln, Cys-Asp-Cys-Arg-Gly-Asp-Cys-Phe-Cys, Ala-Ala-Asn-Leu or Lys-Ala-Leu-Ala-Leu or conservative substitution variants thereof, wherein each amino acid residue, independently, can be replaced by another residue with identical chemical properties.

7. The compound of claim 1 or 2, wherein the oligopeptide comprises one or more of the following sequences: Ala-Phe-Lys, Ala-Leu-Ala-Leu (SEQ ID No. 1), beta-Ala-Leu-Ala-Leu (SEQ ID No. 2), Ala-Leu-Lys-Leu-Leu (SEQ ID No. 3), Ala-Tyr-Gly-Gly-Phe-Leu (SEQ ID No. 4), His-Ser-Ser-Lys-Leu-Gln-Leu (SEQ ID No. 5), Gly-Pro-Leu-Gly-Ile-Ala-Gly-Gln (SEQ ID No. 6), Cys-Asp-Cys-Arg-Gly-Asp-Cys-Phe-Cys (SEQ ID No. 7), Ala-Ala-Asn-Leu (SEQ ID No 8) and Lys-Ala-Leu-Ala-Leu (SEQ ID No 9).

8. A compound according to claim 1 or 2, wherein said cleavable oligopeptide moiety comprises the following amino acid sequence: Ala-Leu-Ala-Leu.

9. A compound according to claim 1 or 2, wherein the enzyme is at least one peptidase selected from the group of: neprilysin (CALLA or CD10), thimet oligopeptidase (TOP), leukotriene A4 hydrolase, endothelin converting enzymes, ste24 protease, neurolysin, mitochondrial intermediate peptidase, interstitial collagenases, collagenases, stromelysins, macrophage elastase, matrilysin, gelatinases, meprins, procollagen C-endopeptidases, procollagen N-endopeptidases, ADAMs and ADAMTs metalloproteinases, myelin associated metalloproteinases, enamelysin, tumor necrosis factor α-converting enzyme, insulysin, nardilysin, mitochondrial processing peptidase, magnolysin, dactylysin-like metalloproteases, neutrophil collagenase, matrix metallopeptidases, membrane-type matrix metalloproteinases, SP2 endopeptidase, prostate specific antigen (PSA), plasmin, urokinase, human fibroblast activation protein (FAPα), trypsin, chymotrypsins, caldecrin, pancreatic elastases, pancreatic endopeptidase, enteropeptidase, leukocyte elastase, myeloblastin, chymases, tryptase, granzyme, stratum corneum chymotryptic enzyme, acrosin, kallikreins, complement components and factors, alternative-complement pathway c3/c5 convertase, mannose-binding protein-associated serine protease, coagulation factors, thrombin, protein c, u and t-type plasminogen activator, cathepsin G, hepsin, prostasin, hepatocyte growth factor-activating endopeptidase, subtilisin/kexin type proprotein convertases, furin, proprotein convertases, prolyl peptidases, acylaminoacyl peptidase, peptidyl-glycaminase, signal peptidase, n-terminal nucleophile aminohydrolases, 20s proteasome, γ-glutamyl transpeptidase, mitochondrial endopeptidase, mitochondrial endopeptidase Ia, htra2 peptidase, matriptase, site 1 protease, legumain, cathepsins, cysteine cathepsins, calpains,

ubiquitin isopeptidase T, caspases, glycosylphosphatidylinositol:protein transamidase, cancer procoagulant, prohormone thiol protease, γ-Glutamyl hydrolase, bleomycin hydrolase, seprase, cathepsin D, pepsins, chymosyn, gastricsin, renin, yapsin and/or memapsins.

10. A compound according to any of the preceding claims, wherein the therapeutic agent is selected from the following group: a chemical agent, a polypeptide, a protein, an antibody a nucleic acid, an antibiotic, or a virus.

11. A compound according to claim 10, wherein the therapeutic agent has anti-tumor therapeutic activity, anti-angiogenic activity or anti-inflammatory activity.

12. A compound according to one of claims 10 or 11, wherein the therapeutic agent is selected from the group of: vincristine, vinblastine, vindesine, vinorelbine, paclitaxel, docetaxel, 10-deacetyltaxol, 7-*epi*-taxol, baccatin III, 7-xylosyltaxol, isotaxel, ifosfamide, colchicine melphalan, chloroaminophene, procarbazine, chlorambucil, thiophosphoramide, busulfan, dacarbazine (DTIC), mitomycins, geldanamycin, nitroso-ureas, estramustine, BCNU, CCNU, fotemustine, streptonigrin, cisplatin, carboplatin, oxaliplatin, methotrexate, aminopterin, 5-fluorouracil, 6-mercaptopurine, raltitrexed, cytosine arabinoside, adenosine arabinoside, gemcitabine, cladribine, clofarabine, pentostatin, fludarabine phosphate, hydroxyureas, camptothecin, irinotecan, topotecan, 9-dimethylaminomethyl-hydroxy-camptothecin hydrochloride, etoposide, teniposide, amsacrine; mitoxantrone; L-canavanine, doxorubicin, THP-adriamycin, daunorubicin, idarubicin, rubidazone, pirarubicin, zorubicin, aclarubicin, epiadriamycin (4'epi-adriamycin or epirubicin), mitoxantrone, bleomycins, actinomycins including actinomycin D, streptozotocin, calicheamycin, duocarmycins, combretastatin; L-asparaginase; hormones; pure inhibitors of aromatase; androgens, analogs-antagonists of LH-RH; interferon alpha (IFN-alpha), interferon gamma (IFN-gamma), interleukin 1 (IL-1), IL-2, IL-4, IL-6, IL-10, IL-12, IL-15, tumor necrosis factor-alpha (TNF-alpha), IGF-1 antagonists (insulin-like growth factor); proteasome inhibitors; farnesyl-transferase inhibitors (FTI); epothilones; maytansinoids; discodermolide; fostriecin; inhibitors of tyrosine kinases such as STI 571 (imatinib mesylate); receptor tyrosine kinase inhibitors

such as erlotinib, sorafenib, vandetanib, canertinib, PKI166, gefitinib, sunitinib, lapatinib, EKB-569; Bcr-Abl kinase inhibitors such as dasatinib, nilotinib, imatinib; aurora kinase inhibitors such as VX-680, CYC116, PHA-739358, SU-6668, JNJ-7706621, MLN8054, AZD-1152, PHA-680632; CDK inhibitors such as flavopirodol, seliciclib, E7070, BMS-387032; MEK inhibitors such as PD184352, U-0126; mTOR inhibitors such as CCI-779 or AP23573; Kinesin spindle inhibitors such as ispinesib or MK-0731; RAF/MEK inhibitors such as Sorafenib, CHIR-265, PLX-4032, CI-1040, PD0325901 or ARRY-142886; bryostatin; L-779450; LY333531; endostatins; proteins; peptides; antibodies; and anti-inflammatory cytokines; the HSP 90 binding agent geldanamycin, macrocyclic polyethers such as halichondrin B, eribulin.

13. A compound according to claim 12, wherein the therapeutic agent is selected from the group of: doxorubicin, paclitaxel, docetaxel, isotaxel, ispinesib, geldanamycin, vinblastine, colchicine, mitomycin C and derivatives thereof.

14. A compound according to claim 1, wherein L1 and L2 each contain two organic functional groups the first organic functional group selected from an amine, hydroxyl or thiol group and the second organic functional group selected from an amine, hydroxyl, thiol, carboxyl, carbonyl, halide or maleimide group and wherein the two functional groups are attached to a backbone comprising from 0 to 50 carbons and heteroatoms (N, O, S), arranged in a straight or branched chain or cyclic moiety or any combination thereof, saturated or unsaturated.

15. A compound according to claim 1, wherein the linking moiety is selected from the group comprising diamino-alkyl, diamino-alkene, diamino-aryl, diamino-cycloalkyl, diamino-poly(ethyleneoxy), aminoalkanoic acid, aminoaryl-carboxylic acid, aminocycloalkyl-carboxylic acid, amino-poly(ethyleneoxy)-carboxylic acid, hydroxylamino-alkyl, hydroxylamino-alkene, hydroxylamino-aryl, hydroxylamino-cycloalkyl, hydroxyl-poly(ethyleneoxy)amine, thioamino-alkyl, thioamino-aryl, thioamino-cycloalkyl, alkanoyl halide, aminoalkyl halide, maleimidoalkanoic acid, maleimidocycloalkyl-carboxylic acid and maleimidoamino alkyl.

16. A compound according to claim 1, wherein the linking moiety L_2 is selected from the group comprising *N,N'*-ethylenediamine, ethanolamine, aminocaproic acid, 4-aminobenzyl alcohol, 6-amino-1-hexanol, 1,3-diaminopropane, cysteamine, 1,4-diaminobutane, *N*-methyl-1,3-propanediamine, *N*-methylethylenediamine and 4-aminobenzylamine.

17. A pharmaceutical composition comprising in a pharmaceutically acceptable carrier at least one compound as defined in any one of the claims 1 to 16.

18. The pharmaceutical composition according to claim 17, intended for an intra-cranial, intra-spinal, enteral or parenteral administration.

19. The pharmaceutical composition according to claim 17 or 18, for the treatment of cancers.

20. The pharmaceutical composition according to claim 19, for the treatment of breast cancers, colorectal cancers, liver cancers, lung cancers such as small cell, non-small cell, bronchic cancers, prostate cancers, ovarian cancers, brain cancers, and pancreatic cancers, colon cancers, head and neck cancers, stomach cancers, bladder cancers, non-Hodgkin's lymphomas, melanomas, leukaemias, neuroblastomas, or glioblastomas.

21. Diagnostic kit comprising a compound according to claim 1 in which D is a marker.

22 A use of an effective amount of at least one compound as defined by one of claims 1 to 16 for the preparation of a pharmaceutical composition for the treatment of cancers or infectious diseases.

23 A pharmaceutically acceptable salt of a compound according to claims 1 to 16.