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(54) **Title**: BIO-ORTHOGONAL DRUG ACTIVATION

(57) **Abstract**: The invention relates to a Prodrug activation method, for therapeutics, wherein use is made of abiotic reactive chemical groups that exhibit bio-orthogonal reactivity towards each other. The invention also relates to a Prodrug kit comprising at least one Prodrug and at least one Activator, wherein the Prodrug comprises a Drug and a first Bio-orthogonal Reactive Group (the Trigger), and wherein the Activator comprises a second Bio-orthogonal Reactive Group. The invention also relates to targeted therapeutics used in the above-mentioned method and kit. The invention particularly pertains to antibody-drug conjugates and to bi- and trispecific antibody derivatives.

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BIO-ORTHOGONAL DRUG ACTIVATION

Field of the Invention

The invention relates to therapeutical methods on the basis of inactivated drugs, such as prodrugs, that are activated by means of an abiotic, bio-orthogonal chemical reaction.

Background of the Invention

In the medical arena the use of inactive compounds such as prodrugs which are activated in a specific site in the human or animal body is well known. Also targeted delivery of inactives such as prodrugs has been studied extensively. Much effort has been devoted to drug delivery systems that effect drug release selectivity at a target site and/or at a desired moment in time. One way is to selectively activate a (systemic) prodrug specifically by local and specific enzymatic activity. However, in many cases a target site of interest lacks a suitable overexpressed enzyme. An alternative is to transport an enzyme to target tissue via a technique called antibody-directed enzyme prodrug therapy (ADEPT). In this approach an enzyme is targeted to a tumor site by conjugation to an antibody that binds a tumor-associated antigen. After systemic administration of the conjugate, its localization at the target and clearance of unbound conjugate, a designed prodrug is administered systemically and locally activated. This method requires the catalysis of a reaction that must not be accomplished by an endogenous enzyme. Enzymes of non-mammalian origin that meet these needs are likely to be highly immunogenic, a fact that makes repeated administration impossible. Alternatively, prodrugs can be targeted to a disease site followed by disease-specific or -non-specific endogenous activation processes (eg pH, enzymes, thiol-containing compounds).

Targeted anticancer therapeutics are designed to reduce nonspecific toxicities and increase efficacy relative to conventional cancer chemotherapy. This approach is embodied by the powerful targeting ability of monoclonal antibodies (mAbs) to specifically deliver highly potent, conjugated small molecule therapeutics to a cancer cell. In an attempt to address the issue of toxicity, chemotherapeutic agents (drugs) have been coupled to targeting molecules such as antibodies or protein receptor ligands that bind with a high degree of specificity to tumor cell to form compounds referred to as antibody-drug conjugates (ADC) or immunoconjugates. Immunoconjugates in theory should be less toxic

because they direct the cytotoxic drug to tumors that express the particular cell surface antigen or receptor. This strategy has met limited success in part because cytotoxic drugs tend to be inactive or less active when conjugated to large antibodies, or protein receptor ligands. Promising advancements with immunoconjugates has seen cytotoxic drugs linked to antibodies through a linker that is cleaved at the tumor site or inside tumor cells (Senter et al, *Current Opinion in Chemical Biology* **2010**, 14:529–537). Ideally, the mAb will specifically bind to an antigen with substantial expression on tumor cells but limited expression on normal tissues. Specificity allows the utilization of drugs that otherwise would be too toxic for clinical application. Most of the recent work in this field has centered on the use of highly potent cytotoxic agents. This requires the development of linker technologies that provide conditional stability, so that drug release occurs after tumor binding, rather than in circulation.

As a conjugate the drug is inactive but upon target localization the drug is released by eg pH or an enzyme, which could be target specific but may also be more generic. The drug release may be achieved by an extracellular mechanism such as low pH in tumor tissue, hypoxia, certain enzymes, but in general more selective drug release can be achieved through intracellular, mostly lysosomal, release mechanisms (e.g. glutathione, proteases, catabolism) requiring the antibody conjugate to be first internalized. Specific intracellular release mechanisms (eg glutathione, cathepsin) usually result in the parent drug, which depending on its properties, can escape the cell and attack neighboring cells. This is viewed as an important mechanism of action for a range of antibody-drug conjugates, especially in tumors with heterogeneous receptor expression, or with poor mAb penetration. Examples of cleavable linkers are: hydrazones (acid labile), peptide linkers (cathepsin B cleavable), hindered disulfide moieties (thiol cleavable). Also non-cleavable linkers can be used in mAb-drug conjugates. These constructs release their drug upon catabolism, presumably resulting in a drug molecule still attached to one amino acid. Only a subset of drugs will regain their activity as such a conjugate. Also, these aminoacid-linked drugs cannot escape the cells. Nevertheless, as the linker is stable, these constructs are generally regarded as the safest and depending on the drug and target, can be very effective.

The current antibody-drug conjugate release strategies have their limitations. The extracellular drug release mechanisms are usually too unspecific (as with pH sensitive linkers) resulting in toxicity. Intracellular release depends on efficient (e.g receptor-mediated internalization) of the mAb-drug, while several cancers lack cancer-specific and efficiently internalizing targets that are present in sufficiently high copy numbers. Intracellular release

may further depend on the presence of an activating enzyme (proteases) or molecules (thiols such as glutathione) in sufficiently high amount. Following intracellular release, the drug may, in certain cases, escape from the cell to target neighbouring cells. This effect is deemed advantageous in heterogeneous tumors where not every cell expresses sufficiently high amounts of target receptor. It is of further importance in tumors that are difficult to penetrate due e.g. to elevated interstitial pressure, which impedes convectional flow. This is especially a problem for large constructs like mAb (conjugates). This mechanism is also essential in cases where a binding site barrier occurs. Once a targeted agent leaves the vasculature and binds to a receptor, its movement within the tumor will be restricted. The likelihood of a mAb conjugate being restricted in the perivascular space scales with its affinity for its target. The penetration can be improved by increasing the mAb dose, however, this approach is limited by dose limiting toxicity in e.g. the liver. Further, antigens that are shed from dying cells can be present in the tumor interstitial space where they can prevent mAb-conjugates of binding their target cell. Also, many targets are hampered by ineffective internalization, and different drugs cannot be linked to a mAb in the same way. Further, it has been proven cumbersome to design linkers to be selectively cleavable by endogenous elements in the target while stable to endogenous elements *en route* to the target (especially the case for slow clearing full mAbs). As a result, the optimal drug, linker, mAb, and target combination needs to be selected and optimized on a case by case basis.

Another application area that could benefit from an effective prodrug approach is the field of T-cell engaging antibody constructs (e.g., bi- or trispecific antibody fragments),which act on cancer by engaging the immunesystem. It has long been considered that bringing activated T-cells into direct contact with cancer cells offers a potent way of killing them (Thompson et al., Biochemical and Biophysical Research Communications 366 (2008) 526–531). Of the many bispecific antibodies that have been created to do this, the majority are composed of two antibody binding sites, one site targets the tumor and the other targets a T-cell (Thakur et al. Current Opinion in Molecular Therapeutics 2010, 12(3), 340-349). However, with bispecific antibodies containing an active T-cell binding site, peripheral T-cell binding will occur. This not only prevents the conjugate from getting to the tumor but can also lead to cytokine storms and T-cell depletion. Photo-activatable anti-T-cell antibodies, in which the anti-T-cell activity is only restored when and where it is required (i.e. after tumor localization via the tumor binding arm), following irradiation with UV light, has been used to overcome these problems. Anti-human CD3 (T-cell targeting) antibodies could be reversibly inhibited with a photocleavable 1-(2-nitrophenyl)ethanol (NPE) coating

(Thompson et al., Biochemical and Biophysical Research Communications 366 (2008) 526–531). However, light based activation is limited to regions in the body where light can penetrate, and is not easily amendable to treating systemic disease such as metastatic cancer. Strongly related constructs that could benfit from a prodrug approach are trispecific T-cell engaging antibody constructs with for example a CD3-and a CD28 T-cell engaging moiety in addition to a cancer targeting agent. Such constructs are too toxic to use as such and either the CD3 or the CD28 or both binding domains need to be masked.

It is desirable to be able to activate targeted drugs selectively and predictably at the target site without being dependent on homogenous penetration and targeting, and on endogenous parameters which may vary *en route* to and within the target, and from indication to indication and from patient to patient.

In order to avoid the drawbacks of current prodrug activation, it has been proposed in Bioconjugate Chem 2008, 19, 714-718, to make use of an abiotic, bio-orthogonal chemical reaction, viz. the Staudinger reaction, to provoke activation of the prodrug. Briefly, in the introduced concept, the Prodrug is a conjugate of a Drug and a Trigger, and this Drug-Trigger conjugate is not activated endogeneously by e.g. an enzyme or a specific pH, but by a controlled administration of the Activator, i.e. a species that reacts with the Trigger moiety in the Prodrug, to induce release of the Drug from the Trigger (or vice versa, release of the Trigger from the Drug, however one may view this release process). The presented Staudinger approach for this concept, however, has turned out not to work well, and its area of applicability is limited in view of the specific nature of the release mechanism imposed by the Staudinger reaction. Other drawbacks for use of Staudinger reactions are their limited reaction rates, and the oxidative instability of the phosphine components of these reactions. Therefore, it is desired to provide reactants for an abiotic, bio-orthogonal reaction that are stable in physiological conditions, that are more reactive towards each other, and that are capable of inducing release of a bound drug by means of a variety of mechanisms, thus offering a greatly versatile activated drug release method.

The use of a biocompatible chemical reaction that does not rely on endogenous activation mechanisms (eg pH, enzymes) for selective Prodrug activation would represent a powerful new tool in cancer therapy. Selective activation of Prodrugs when and where required allows control over many processes within the body, including cancer. Therapies, such as anti-tumor antibody therapy, may thus be made more specific, providing an increased therapeutic contrast between normal cells and tumour to reduce unwanted side effects. In the context of T-cell engaging anticancer antibodies, the present invention allows

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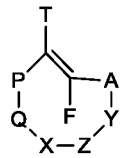
the systemic administration and tumor targeting of an inactive antibody construct (i.e. this is then the Prodrug), diminishing off-target toxicity. Upon sufficient tumor uptake and clearance from non target areas, the tumor-bound antibody is activated by administration of the Activator, which reacts with the Trigger or Triggers on the antibody or particular antibody domain, resulting in removal of the Trigger and restoration of the T-cell binding function. This results in T-cell activation and anticancer action (i.e. this is then the Drug release).

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed in Australia before the priority date of each claim of this application.

Summary of the Invention

In order to better address one or more of the foregoing desires, disclosed herein is a kit for the administration and activation of a Prodrug, the kit comprising a Drug linked, directly or indirectly, to a Trigger moiety, and an Activator for the Trigger moiety, wherein the Trigger moiety comprises a dienophile and the Activator comprises a diene, the dienophile satisfying the following formula (1a):



(1a)

wherein T, F each independently denotes H, or a substituent selected from the group consisting of alkyl, F, Cl, Br or I; the meaning of the letters A,P,Q,X,Y, and Z is selected from the group consisting of the following Embodiments:

(1) one of the bonds PQ, QP, QX, XQ, XZ, ZX, ZY, YZ, YA, AY consists of -CR^aX^D-CR^aY^D-, the remaining groups constituted by A,Y,Z,X,Q,P being independently from each other CR^a_2, S, O, SiR^b_2, such that P and A are CR^a_2, and no adjacent pairs of atoms are present selected from the group consisting of O-O, O-S, and S-S, and such that Si, if present, is adjacent to CR^a_2 or O; X^D is O-C(O)-$(L^D)_n$-(D^D), S-C(O)-$(L^D)_n$-(D^D), O-C(S)-$(L^D)_n$-(D^D), S-C(S)-$(L^D)_n$-(D^D), NR^c-C(O)-$(L^D)_n$-(D^D), NR^c-C(S)-$(L^D)_n$-(D^D); and Y^D is NHR^c, OH, SH;

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or X^D is C(O)-$(L^D)_n$-(D^D), C(S)-$(L^D)_n$-(D^D); and Y^D is $CR^c_2NHR^c$, CR^c_2OH, CR^c_2SH, NH-NH_2, O-NH_2, or NH-OH;

(2) A is CR^aX^D and Z is CR^aY^D, or Z is CR^aX^D and A is CR^aY^D, or P is CR^aX^D and X is CR^aY^D, or X is CR^aX^D and P is CR^aY^D, such that X^D and Y^D are positioned in a *trans* conformation with respect to one another; the remaining groups constituted by A,Y,Z,X,Q, and P being independently from each other CR^a_2, S, O, SiR^b_2, such that P and A are CR^a_2, and no adjacent pairs of atoms are present selected from the group consisting of O-O, O-S, and S-S, and such that Si, if present, is adjacent to CR^a_2 or O; X^D is O-C(O)-$(L^D)_n$-(D^D), S-C(O)-$(L^D)_n$-(D^D), O-C(S)-$(L^D)_n$-(D^D), S-C(S)-$(L^D)_n$-(D^D), NR^c-C(O)-$(L^D)_n$-(D^D), NR^c-C(S)-$(L^D)_n$-(D^D), and Y^D is NHR^c, OH, SH, $CR^c_2NHR^c$, CR^c_2OH, CR^c_2SH, NH-NH_2, O-NH_2, or NH-OH; or X^D is CR^c_2-O-C(O)-$(L^D)_n$-(D^D), CR^c_2-S-C(O)-$(L^D)_n$-(D^D), CR^c_2-O-C(S)-$(L^D)_n$-(D^D), CR^c_2-S-C(S)-$(L^D)_n$-(D^D), CR^c_2-NR^c-C(O)-$(L^D)_n$-(D^D), CR^c_2-NR^c-C(S)-$(L^D)_n$-(D^D), and Y^D is NHR^c, OH, SH; or X^D is C(O)-$(L^D)_n$-(D^D), C(S)-$(L^D)_n$-(D^D); and Y^D is $CR^c_2NHR^c$, CR^c_2OH, CR^c_2SH, NH-NH_2, O-NH_2, NH-OH.

(3) A is CR^aY^D and one of P, Q, X, Z is CR^aX^D, or P is CR^aY^D and one of A, Y, Z, X is CR^aX^D, or Y is CR^aY^D and X or P is CR^aX^D, or Q is CR^aY^D and Z or A is CR^aX^D, or either Z or X is CR^aY^D and A or P is CR^aX^D, such that X^D and Y^D are positioned in a *trans* conformation with respect to one another; the remaining groups constituted by A,Y,Z,X,Q, and P being independently from each other CR^a_2, S, O, SiR^b_2, such that P and A are CR^a_2, and no adjacent pairs of atoms are present selected from the group consisting of O-O, O-S, and S-S, and such that Si, if present, is adjacent to CR^a_2 or O; X^D is $(O\text{-}C(O))_p$-$(L^D)_n$-(D^D), S-C(O)-$(L^D)_n$-(D^D), O-C(S)-$(L^D)_n$-(D^D), S-C(S)-$(L^D)_n$-(D^D), and Y^D is $CR^c_2NHR^c$, CR^c_2OH, CR^c_2SH, NH-NH_2, O-NH_2, NH-OH; with p being 0 or 1;

(4) P is CR^aY^D and Y is CR^aX^D, or A is CR^aY^D and Q is CR^aX^D, or Q is CR^aY^D and A is CR^aX^D, or Y is CR^aY^D and P is CR^aX^D, such that X^D and Y^D are positioned in a *trans* conformation with respect to one another; the remaining groups constituted from A,Y,Z,X,Q, and P being independently from each other CR^a_2, S, O, SiR^b_2, such that P and A are CR^a_2, and no adjacent pairs of atoms are present selected from the group consisting of O-O, O-S, and S-S, and such that Si, if present, is adjacent to CR^a_2 or O; X^D is $(O\text{-}C(O))_p$-$(L^D)_n$-(D^D), S-C(O)-$(L^D)_n$-(D^D), O-C(S)-$(L^D)_n$-(D^D), S-C(S)-$(L^D)_n$-(D^D); Y^D is NHR^c, OH, SH; p = 0 or 1.

(5) Y is Y^D and P is CR^aX^D, or Q is Y^D and A is CR^aX^D; the remaining groups constituted by A,Y,Z,X,Q, and P being independently from each other CR^a_2, S, O, SiR^b_2, such that P and A are CR^a_2, and no adjacent pairs of atoms are present selected from the

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group consisting of O-O, O-S, and S-S, and such that Si, if present, is adjacent to CR^a_2 or O; X^D is $(O\text{-}C(O))_p\text{-}(L^D)_n\text{-}(D^D)$, $S\text{-}C(O)\text{-}(L^D)_n\text{-}(D^D)$, $O\text{-}C(S)\text{-}(L^D)_n\text{-}(D^D)$, $S\text{-}C(S)\text{-}(L^D)_n\text{-}(D^D)$, $NR^c\text{-}C(O)\text{-}(L^D)_n\text{-}(D^D)$, $NR^c\text{-}C(S)\text{-}(L^D)_n\text{-}(D^D)$, $C(O)\text{-}(L^D)_n\text{-}(D^D)$, $C(S)\text{-}(L^D)_n\text{-}(D^D)$; Y^D is NH; p = 0 or 1;

(6) Y is Y^D and P or Q is X^D, or Q is Y^D and A or Y is X^D; the remaining groups constituted by A,Y,Z,X,Q, and P being independently from each other CR^a_2, S, O, SiR^b_2, such that P and A are CR^a_2, and no adjacent pairs of atoms are present selected from the group consisting of O-O, O-S, and S-S, and such that Si, if present, is adjacent to CR^a_2 or O; X^D is $N\text{-}C(O)\text{-}(L^D)_n\text{-}(D^D)$, $N\text{-}C(S)\text{-}(L^D)_n\text{-}(D^D)$; Y^D is NH;

wherein each R^a independently is selected from the group consisting of H, alkyl, aryl, OR', SR', S(=O)R''', $S(=O)_2R'''$, $S(=O)_2NR'R''$, Si-R''', Si-O-R''', OC(=O)R''', SC(=O)R''', OC(=S)R''', SC(=S)R''', F, Cl, Br, I, N_3, SO_2H, SO_3H, SO_4H, PO_3H, PO_4H, NO, NO_2, CN, OCN, SCN, NCO, NCS, CF_3, CF_2-R', NR'R", C(=O)R', C(=S)R', C(=O)O-R', C(=S)O-R', C(=O)S-R', C(=S)S-R', C(=O)NR'R", C(=S)NR'R'', NR'C(=O)-R''', NR'C(=S)-R''', NR'C(=O)O-R''', NR'C(=S)O-R''', NR'C(=O)S-R''', NR'C(=S)S-R''', OC(=O)NR'-R''', SC(=O)NR'-R''', OC(=S)NR'-R''', SC(=S)NR'-R''', NR'C(=O)NR"-R'', NR'C(=S)NR"-R'', CR'NR'', with each R' and each R'' independently being H, aryl or alkyl and R''' independently being aryl or alkyl; wherein each R^b independently is selected from the group consisting of H, alkyl, aryl, O-alkyl, O-aryl, OH; wherein each R^c is independently selected from H, $C_{1\text{-}6}$ alkyl and $C_{1\text{-}6}$ aryl;

wherein two or more $R^{a,b,c}$ moieties together may form a ring;

and wherein $(L^D)_n$ is an optional linker with n= 0 or 1, preferably linked to T^R via S, N, NH, or O, wherein these atoms are part of the linker, which may consist of multiple units arranged linearly and/or branched; D^D is one or more therapeutic moieties or drugs, preferably linked via S, N, NH, or O, wherein these atoms are part of the therapeutic moiety.

Also disclosed herein is a Prodrug comprising a Drug compound linked, directly or indirectly, to a *trans*-cyclooctene moiety satisfying the above formula (1a).

Also disclosed herein is a method of modifying a Drug compound into a Prodrug that can be triggered by an abiotic, bio-orthogonal reaction, the method comprising the steps of providing a Drug and chemically linking the Drug to a cyclic moiety satisfying the above formula (1a).

Also disclosed herein is a method of treatment wherein a patient suffering from a disease that can be modulated by a drug, is treated by administering, to said patient, a Prodrug comprising a Trigger moiety after activation of which by administration of an

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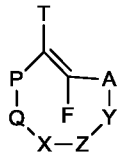
Activator the Drug will be released, wherein the Trigger moiety comprises a ring structure satisfying the above formula (1a).

Also disclosed herein is a compound comprising an eight-membered non-aromatic cyclic mono-alkenylene moiety (preferably a cyclooctene moiety, and more preferably a *trans*-cyclooctene moiety), said moiety comprising a linkage to a Drug, for use in prodrug therapy in an animal or a human being.

Also disclosed herein is the use of a diene, preferably a tetrazine, as an activator for the release, in a physiological environment, of a substance linked to a compound satisfying formula (1a). In connection herewith, the invention also pertains to a tetrazine for use as an activator for the release, in a physiological environment, of a substance linked to a a compound satisfying formula (1a), and to a method for activating, in a physiological environment, the release of a substance linked to a compound satisfying formula (1a), wherein a tetrazine is used as an activator.

Also disclosed herein is the use of the inverse electron-demand Diels-Alder reaction between a compound satisfying formula (1a) and a diene, preferably a tetrazine, as a chemical tool for the release, in a physiological environment, of a substance administered in a covalently bound form, wherein the substance is bound to a compound satisfying formula (1a).

In a first aspect, there is provided a kit for the administration and activation of a Prodrug, the kit comprising a Drug D^D linked, directly or indirectly, to a Trigger moiety T^R, and an Activator for the Trigger moiety, wherein the Trigger moiety comprises a dienophile and the Activator comprises a diene, the dienophile, including said Drug linked thereto, satisfying the following formula (1a):



(1a)

wherein T, F each independently denotes H, or a substituent selected from the group consisting of alkyl, F, Cl, Br or I; the meaning of the letters A,P,Q,X,Y, and Z is selected from the group consisting of the following (1) to (6):

(1) one of the bonds PQ, QP, QX, XQ, XZ, ZX, ZY, YZ, YA, AY consists of -CR^aX^D-CR^aY^D-, the remaining groups constituted by A,Y,Z,X,Q,P being independently from each other CR^a_2, S, O, SiR^b_2, such that P and A are CR^a_2, and no adjacent pairs of atoms are present selected from the group consisting of O-O, O-S, and S-S, and such that Si, if present,

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is adjacent to CR^a_2 or O; X^D is O-C(O)-$(L^D)_n$-(D^D), S-C(O)-$(L^D)_n$-(D^D), O-C(S)-$(L^D)_n$-(D^D), S-C(S)-$(L^D)_n$-(D^D), NR^c-C(O)-$(L^D)_n$-(D^D), NR^c-C(S)-$(L^D)_n$-(D^D); and Y^D is NHR^c, OH, SH; or X^D is C(O)-$(L^D)_n$-(D^D), C(S)-$(L^D)_n$-(D^D); and Y^D is $CR^c_2NHR^c$, CR^c_2OH, CR^c_2SH, NH-NH_2, O-NH_2, or NH-OH;

(2) A is CR^aX^D and Z is CR^aY^D, or Z is CR^aX^D and A is CR^aY^D, or P is CR^aX^D and X is CR^aY^D, or X is CR^aX^D and P is CR^aY^D, such that X^D and Y^D are positioned in a *trans* conformation with respect to one another; the remaining groups constituted by A,Y,Z,X,Q, and P being independently from each other CR^a_2, S, O, SiR^b_2, such that P and A are CR^a_2, and no adjacent pairs of atoms are present selected from the group consisting of O-O, O-S, and S-S, and such that Si, if present, is adjacent to CR^a_2 or O; X^D is O-C(O)-$(L^D)_n$-(D^D), S-C(O)-$(L^D)_n$-(D^D), O-C(S)-$(L^D)_n$-(D^D), S-C(S)-$(L^D)_n$-(D^D), NR^c-C(O)-$(L^D)_n$-(D^D), NR^c-C(S)-$(L^D)_n$-(D^D), and Y^D is NHR^c, OH, SH, $CR^c_2NHR^c$, CR^c_2OH, CR^c_2SH, NH-NH_2, O-NH_2, or NH-OH; or X^D is CR^c_2-O-C(O)-$(L^D)_n$-(D^D), CR^c_2-S-C(O)-$(L^D)_n$-(D^D), CR^c_2-O-C(S)-$(L^D)_n$-(D^D), CR^c_2-S-C(S)-$(L^D)_n$-(D^D), CR^c_2-NR^c-C(O)-$(L^D)_n$-(D^D), CR^c_2-NR^c-C(S)-$(L^D)_n$-(D^D), and Y^D is NHR^c, OH, SH; or X^D is C(O)-$(L^D)_n$-(D^D), C(S)-$(L^D)_n$-(D^D); and Y^D is $CR^c_2NHR^c$, CR^c_2OH, CR^c_2SH, NH-NH_2, O-NH_2, NH-OH.

(3) A is CR^aY^D and one of P, Q, X, Z is CR^aX^D, or P is CR^aY^D and one of A, Y, Z, X is CR^aX^D, or Y is CR^aY^D and X or P is CR^aX^D, or Q is CR^aY^D and Z or A is CR^aX^D, or either Z or X is CR^aY^D and A or P is CR^aX^D, such that X^D and Y^D are positioned in a *trans* conformation with respect to one another; the remaining groups constituted by A,Y,Z,X,Q, and P being independently from each other CR^a_2, S, O, SiR^b_2, such that P and A are CR^a_2, and no adjacent pairs of atoms are present selected from the group consisting of O-O, O-S, and S-S, and such that Si, if present, is adjacent to CR^a_2 or O; X^D is $(O\text{-}C(O))_p$-$(L^D)_n$-(D^D), S-C(O)-$(L^D)_n$-(D^D), O-C(S)-$(L^D)_n$-(D^D), S-C(S)-$(L^D)_n$-(D^D), and Y^D is $CR^c_2NHR^c$, CR^c_2OH, CR^c_2SH, NH-NH_2, O-NH_2, NH-OH; with p being 0 or 1;

(4) P is CR^aY^D and Y is CR^aX^D, or A is CR^aY^D and Q is CR^aX^D, or Q is CR^aY^D and A is CR^aX^D, or Y is CR^aY^D and P is CR^aX^D, such that X^D and Y^D are positioned in a *trans* conformation with respect to one another; the remaining groups constituted from A,Y,Z,X,Q, and P beng independently from each other CR^a_2, S, O, SiR^b_2, such that P and A are CR^a_2, and no adjacent pairs of atoms are present selected from the group consisting of O-O, O-S, and S-S, and such that Si, if present, is adjacent to CR^a_2 or O; X^D is $(O\text{-}C(O))_p$-$(L^D)_n$-(D^D), S-C(O)-$(L^D)_n$-(D^D), O-C(S)-$(L^D)_n$-(D^D), S-C(S)-$(L^D)_n$-(D^D); Y^D is NHR^c, OH, SH; p = 0 or 1.

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(5) Y is Y^D and P is CR^aX^D, or Q is Y^D and A is CR^aX^D; the remaining groups constituted by A,Y,Z,X,Q, and P being independently from each other CR^a_2, S, O, SiR^b_2, such that P and A are CR^a_2, and no adjacent pairs of atoms are present selected from the group consisting of O-O, O-S, and S-S, and such that Si, if present, is adjacent to CR^a_2 or O; X^D is $(O\text{-}C(O))_p\text{-}(L^D)_n\text{-}(D^D)$, $S\text{-}C(O)\text{-}(L^D)_n\text{-}(D^D)$, $O\text{-}C(S)\text{-}(L^D)_n\text{-}(D^D)$, $S\text{-}C(S)\text{-}(L^D)_n\text{-}(D^D)$, $NR^c\text{-}C(O)\text{-}(L^D)_n\text{-}(D^D)$, $NR^c\text{-}C(S)\text{-}(L^D)_n\text{-}(D^D)$, $C(O)\text{-}(L^D)_n\text{-}(D^D)$, $C(S)\text{-}(L^D)_n\text{-}(D^D)$; Y^D is NH; p = 0 or 1;

(6) Y is Y^D and P or Q is X^D, or Q is Y^D and A or Y is X^D; the remaining groups constituted by A,Y,Z,X,Q, and P being independently from each other CR^a_2, S, O, SiR^b_2, such that P and A are CR^a_2, and no adjacent pairs of atoms are present selected from the group consisting of O-O, O-S, and S-S, and such that Si, if present, is adjacent to CR^a_2 or O; X^D is $N\text{-}C(O)\text{-}(L^D)_n\text{-}(D^D)$, $N\text{-}C(S)\text{-}(L^D)_n\text{-}(D^D)$; Y^D is NH;

wherein each R^a independently is selected from the group consisting of H, alkyl, aryl, OR', SR', S(=O)R''', $S(=O)_2R'''$, $S(=O)_2NR'R''$, Si-R''', Si-O-R''', OC(=O)R''', SC(=O)R''', OC(=S)R''', SC(=S)R''', F, Cl, Br, I, N_3, SO_2H, SO_3H, SO_4H, PO_3H, PO_4H, NO, NO_2, CN, OCN, SCN, NCO, NCS, CF_3, CF_2-R', NR'R", C(=O)R', C(=S)R', C(=O)O-R', C(=S)O-R', C(=O)S-R', C(=S)S-R', C(=O)NR'R", C(=S)NR'R'', NR'C(=O)-R''', NR'C(=S)-R''', NR'C(=O)O-R''', NR'C(=S)O-R''', NR'C(=O)S-R''', NR'C(=S)S-R''', OC(=O)NR'-R''', SC(=O)NR'-R''', OC(=S)NR'-R''', SC(=S)NR'-R''', NR'C(=O)NR"-R'', NR'C(=S)NR"-R'', CR'NR'', with each R' and each R'' independently being H, aryl or alkyl and R''' independently being aryl or alkyl; wherein each R^b is independently selected from the group consisting of H, alkyl, aryl, O-alkyl, O-aryl, OH; wherein each R^c is independently selected from H, $C_{1\text{-}6}$ alkyl and $C_{1\text{-}6}$ aryl;

wherein two or more $R^{a,b,c}$ moieties together may form a ring;

$(L^D)_n$ is an optional linker with n= 0 or 1, preferably linked to T^R via S, N, NH, or O, wherein these atoms are part of the linker, which may consist of multiple units arranged linearly and/or branched; D^D is one or more drugs, preferably linked via S, N, NH, or O, wherein these atoms are part of the therapeutic moiety.

In a second aspect, there is provided a prodrug comprising a drug compound linked, directly or indirectly, to a dienophile moiety of formula (1a), as described in the first aspect.

In a third aspect, there is provided a method of modifying a drug compound into a prodrug that can be triggered by an abiotic, bio-orthogonal reaction, comprising

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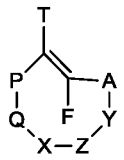
providing a drug and chemically linking the drug to a dienophile moiety, so as to form a prodrug of formula (1a) as described in the first aspect.

In a fourth aspect, there is provided a method of treatment wherein a patient suffering from a disease that can be modulated by a drug, is treated by administering, to said patient, a prodrug comprising a trigger moiety and an activator for the trigger moiety after activation of which the drug will be released, wherein the trigger moiety comprises a *trans*-cyclooctene ring, the ring optionally including one or more hetero-atoms, and the activator comprises a diene being selected so as to be capable of reacting with the dienophile in an inverse electron-demand Diels-Alder reaction, the trigger moiety satisfying the formula (1a) as described in the first aspect.

In a fifth aspect, there is provided a compound satisfying the formula (1a) as described in the first aspect:



(1a)

said compound comprising a linkage to a drug, for use in prodrug therapy in an animal or a human being.

In a sixth aspect, there is provided the use of a tetrazine as an activator for the release, in a physiological environment, of a substance linked to a compound satisfying formula (1a) as described in the first aspect.

In a seventh aspect, there is provided the use of the inverse electron-demand Diels-Alder reaction between a compound satisfying formula (1a) as described in the first aspect and a tetrazine as a chemical tool for the release, in a physiological environment, of a substance administered in a chemically bound form, wherein the substance is bound to a compound satisfying formula (1a).

In an eighth aspect, there is provided the use of a *trans*-cyclooctene satisfying formula (1a), as described in the first aspect, as a carrier for a therapeutic compound.

The inverse electron demand ("retro") Diels-Alder reaction

The dienophile of formula (1a) and the diene are capable of reacting in an inverse electron-demand Diels-Alder reaction. Activation of the Prodrug by the retro Diels-Alder reaction of the Trigger with the Activator leads to release of the Drug.

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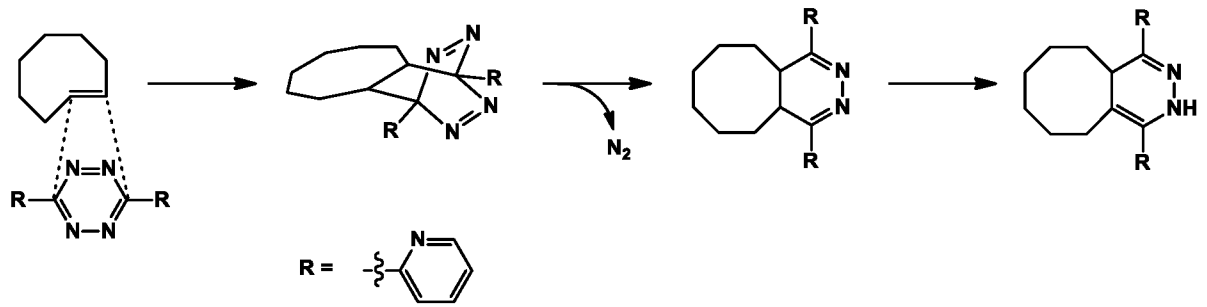
Below a reaction scheme is given for a [4+2] Diels-Alder reaction between the (3,6)-di-(2-pyridyl)-s-tetrazine diene and a trans-cylcooctene dienophile, followed by a retro Diels Alder reaction in which the product and dinitrogen is formed. The reaction product may tautomerize, and this is also shown in the scheme. Because the trans cyclooctene derivative does not contain electron withdrawing groups as in the classical Diels Alder reaction, this type of Diels Alder reaction is distinguished from the classical one, and frequently referred to as an "inverse electron demand Diels Alder reaction". In the following text the sequence of both reaction steps, i.e. the initial Diels-Alder cyclo-addition (typically an inverse electron demand Diels Alder cyclo-addition) and the subsequent retro Diels Alder reaction will be referred to in shorthand as "retro Diels Alder reaction" or "retro-DA". It will sometimes be abbreviated as "rDA" reaction. The product of the reaction is then the retro Diels-Alder adduct, or the rDA adduct.



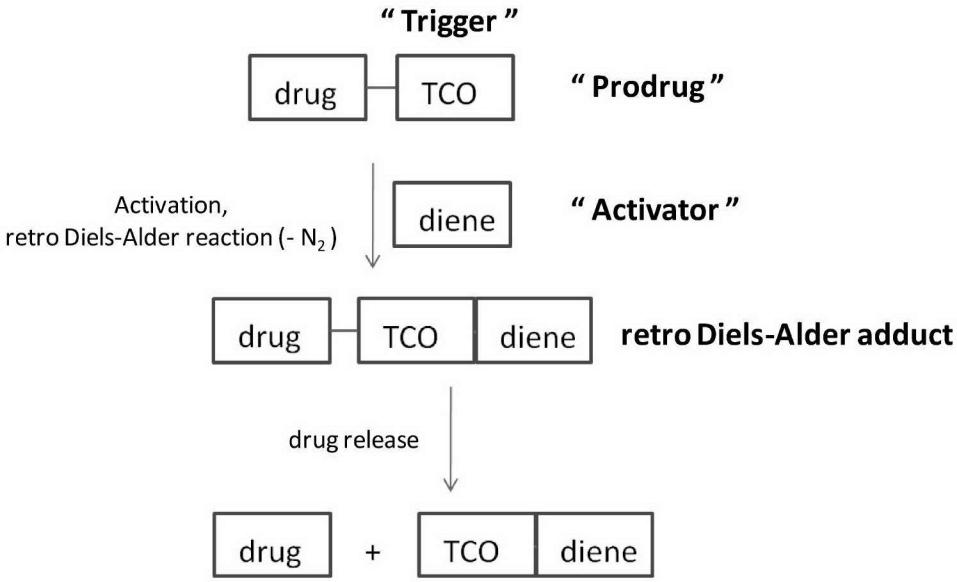
Detailed Description of the Invention

In a general sense, the invention is based on the recognition that a drug can be released from *trans*-cyclooctene derivatives satisfying formula (1a) upon cyclooaddition with compatible dienes, such as tetrazine derivatives. The dienophiles of formula (1a) have the advantage that they react (and effectuate drug release) with substantially any diene .

Without wishing to be bound by theory, the inventors believe that the molecular structure of the retro Diels-Alder adduct is such that a spontaneous elimination or cyclization reaction within this rDA adduct releases the drug. Particularly, the inventors believe that appropriately modified rDA components, i.e. according to the present invention, lead to rDA adducts wherein the bond to the drug on the part originating from the dienophile is broken by the reaction with a nucleophile on the part originating from the dienophile, while such an intramolecular reaction within the part originating from the dienophile is precluded prior to rDA reaction with the diene.

The general concept of using the retro-Diels Alder reaction in Prodrug activation is illustrated in Scheme 1.

Scheme 1:



In this scheme "TCO" stands for *trans*-cyclooctene. The term *trans*-cyclooctene is used here as possibly including one or more hetero-atoms, and particularly refers to a structure satisfying formula (1a). In a broad sense,
the inventors have found that – other than the attempts made on the basis of the Staudinger reaction – the selection of a TCO as the trigger moiety for a prodrug, provides a versatile tool to render drug (active) moieties into prodrug (activatable) moieties, wherein the activation occurs through a powerful, abiotic, bio-orthogonal reaction of the dienophile (Trigger) with the diene (Activator), viz the aforementioned retro Diels-Alder reaction, and wherein the Prodrug is a Drug-dienophile conjugate.

It will be understood that in Scheme 1 in the retro Diels-Alder adduct as well as in the end product, the indicated TCO group and the indicated diene group are the residues of, respectively, the TCO and diene groups after these groups have been converted in the retro Diels-Alder reaction.

A requirement for the successful application of an abiotic bio-orthogonal chemical reaction is that the two participating functional groups have finely tuned reactivity so that interference with coexisting functionality is avoided. Ideally, the reactive partners would be abiotic, reactive under physiological conditions, and reactive only with each other

while ignoring their cellular/physiological surroundings (bio-orthogonal). The demands on selectivity imposed by a biological environment preclude the use of most conventional reactions.

The inverse electron demand Diels Alder reaction, however, has proven utility in animals at low concentrations and semi-equimolar conditions (R. Rossin et al, *Angewandte Chemie Int Ed* 2010, 49, 3375-3378). The reaction partners subject to this invention are strained trans-cyclooctene (TCO) derivatives and suitable dienes, such as tetrazine derivatives. The cycloaddition reaction between a TCO and a tetrazine affords an intermediate, which then rearranges by expulsion of dinitrogen in a retro-Diels–Alder cycloaddition to form a dihydropyridazine conjugate. This and its tautomers is the retro Diels-Alder adduct.

Reflecting the suitability of the rDA reaction, the invention provides, in one aspect, the use of a tetrazine as an activator for the release, in a physiological environment, of a substance linked to a *trans*-cyclooctene. In connection herewith, the invention also pertains to a tetrazine for use as an activator for the release, in a physiological environment, of a substance linked to a *trans*-cyclooctene, and to a method for activating, in a physiological environment, the release of a substance linked to a *trans*-cyclooctene, wherein a tetrazine is uses as an activator.

The present inventors have further come to the non-obvious insight, that the structure of the TCO of formula (1a), *par excellence*, is suitable to provoke the release of a drug linked to it, as a result of the reaction involving the double bond available in the TCO dienophile, and a diene. The feature believed to enable this is the change in nature of the eight membered ring of the TCO in the dienophile reactant as compared to that of the eight membered ring in the rDA adduct. The eight membered ring in the rDA adduct has significantly more conformational freedom and has a significantly different conformation as compared to the eight membered ring in the highly strained TCO prior to rDA reaction. The nucleophilic site in the dienophile prior to rDA reaction is locked within the specific conformation of the dienophile and is therefore not properly positioned to react intramolecularly and to thereby release the drug species. In contrast, and due to the changed nature of the eight membered ring, this nucleophilic site is properly positioned within the rDA adduct and will react intramolecularly, thereby releasing the drug. According to the above, but without being limited by theory, we believe that the drug release is mediated by strain-release of the TCO-dienophile after and due to the rDA reaction with the diene Activator.

. It is to be emphasized that the invention is thus of a scope well beyond specific chemical structures. In a broad sense, the invention puts to use the recognition that the rDA reaction using a dienophile of formula (1a) as well as the rDA adduct embody a versatile platform for enabling provoked drug release in a bioorthogonal reaction.

Reflecting on this, the invention also presents the use of the inverse electron-demand Diels-Alder reaction between a *trans*-cyclooctene and a tetrazine as a chemical tool for the release, in a physiological environment, of a bound substance.

The fact that the reaction is bio-orthogonal, and that many structural options exist for the reaction pairs, will be clear to the skilled person. E.g., the rDA reaction is known in the art of pre-targeted medicine. Reference is made to, e.g., WO 2010/119382, WO 2010/119389, and WO 2010/051530. Whilst the invention presents an entirely different use of the reaction, it will be understood that the various structural possibilities available for the rDA reaction pairs as used in pre-targeting, are also available in the field of the present invention.

The dienophile trigger moiety used in the present invention comprises a *trans*-cyclooctene ring, the ring optionally including one or more hetero-atoms. Hereinafter this eight-membered ring moiety will be defined as a *trans*-cyclooctene moiety, for the sake of legibility, or abbreviated as "TCO" moiety. It will be understood that the essence resides in the possibility of the eight-membered ring to act as a dienophile and to be released from its conjugated drug upon reaction. The skilled person is familiar with the fact that the dienophile activity is not necessarily dependent on the presence of all carbon atoms in the ring, since also heterocyclic monoalkenylene eight-membered rings are known to possess dienophile activity.

Thus, in general, the invention is not limited to strictly drug-substituted *trans*-cyclooctene. The person skilled in organic chemistry will be aware that other eight-membered ring-based dienophiles exist, which comprise the same endocyclic double bond as the *trans*-cyclooctene, but which may have one or more heteroatoms elsewhere in the ring. I.e., the invention generally pertains to eight-membered non-aromatic cyclic alkenylene moieties, preferably a cyclooctene moiety, and more preferably a *trans*-cyclooctene moiety, comprising a conjugated drug.

Other than is the case with e.g. medicinally active substances, where the *in vivo* action is often changed with minor structural changes, the present invention first and foremost requires the right chemical reactivity combined with an appropriate design of the

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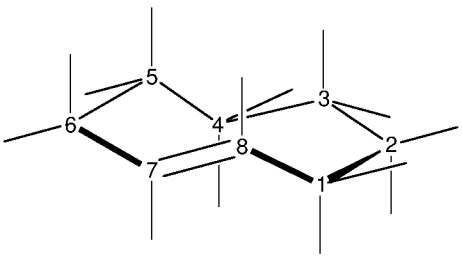
drug-conjugate. Thus, the possible structures extend to those of which the skilled person is familiar with that these are reactive as dienophiles.

It should be noted that, depending on the choice of nomenclature, the TCO dienophile may also be denoted E-cyclooctene. With reference to the conventional nomenclature, it will be understood that, as a result of substitution on the cyclooctene ring, depending on the location and molecular weight of the substituent, the same cyclooctene isomer may formally become denoted as a Z-isomer. In the present invention, any substituted variants of the invention, whether or not formally “E” or “Z,” or “cis” or “trans” isomers, will be considered derivatives of unsubstituted *trans*-cyclooctene, or unsubstituted E-cyclooctene. The terms ”*trans*-cyclooctene” (TCO) as well as E-cyclooctene are used interchangeably and are maintained for all dienophiles according to the present invention, also in the event that substituents would formally require the opposite nomenclature. I.e., the invention relates to cyclooctene in which carbon atoms 1 and 6 as numbered below are in the E (*entgegen*) or *trans* position.



Formula (1)

The present invention will further be described with respect to particular embodiments and with reference to certain drawings but the invention is not limited thereto but only by the claims. Any reference signs in the claims shall not be construed as limiting the scope. The drawings described are only schematic and are non-limiting. In the drawings, the size of some of the elements may be exaggerated and not drawn on scale for illustrative purposes. Where an indefinite or definite article is used when referring to a singular noun e.g. "a" or "an", "the", this includes a plural of that noun unless something else is specifically stated.

It is furthermore to be noticed that the term "comprising", used in the description and in the claims, should not be interpreted as being restricted to the means listed thereafter; it does not exclude other elements or steps. Thus, the scope of the expression "a device comprising means A and B" should not be limited to devices consisting only of

components A and B. It means that with respect to the present invention, the only relevant components of the device are A and B.

In several chemical formulae below reference is made to "alkyl" and "aryl." In this respect "alkyl", each independently, indicates an aliphatic, straight, branched, saturated, unsaturated and/or or cyclic hydrocarbyl group of up to ten carbon atoms, possibly including 1-10 heteroatoms such as O, N, or S, and "aryl", each independently, indicates an aromatic or heteroaromatic group of up to twenty carbon atoms, that possibly is substituted, and that possibly includes 1-10 heteroatoms such as O, N, P or S. "Aryl" groups also include "alkylaryl" or "arylalkyl" groups (simple example: benzyl groups). The number of carbon atoms that an "alkyl", "aryl", "alkylaryl" and "arylalkyl" contains can be indicated by a designation preceding such terms (i.e. C_{1-10} alkyl means that said alkyl may contain from 1 to 10 carbon atoms). Certain compounds of the invention possess chiral centers and/or tautomers, and all enantiomers, diasteriomers and tautomers, as well as mixtures thereof are within the scope of the invention. In several formulae, groups or substituents are indicated with reference to letters such as "A", "B", "X", "Y", and various (numbered) "R" groups. The definitions of these letters are to be read with reference to each formula, i.e. in different formulae these letters, each independently, can have different meanings unless indicated otherwise.

In all embodiments of the invention as described herein, alkyl is preferably lower alkyl (C_{1-4} alkyl), and each aryl preferably is phenyl.

Earlier work (R. Rossin et al, *Angewandte Chemie Int Ed* 2010, 49, 3375-3378) demonstrated the utility of the inverse-electron-demand Diels Alder reaction for pretargeted radioimmunoimaging. This particular cycloaddition example occurred between a (3,6)-di-(2-pyridyl)-s-tetrazine derivative and a E-cyclooctene, followed by a retro Diels Alder reaction in which the product and nitrogen is formed. Because the trans cyclooctene derivative does not contain electron withdrawing groups as in the classical Diels Alder reaction, this type of Diels Alder reaction is distinguished from the classical one, and frequently referred to as an "inverse electron demand Diels Alder reaction". In the following text the sequence of both reaction steps, i.e. the initial Diels-Alder cyclo-addition (typically an inverse electron demand Diels Alder cyclo-addition) and the subsequent retro Diels Alder reaction will be referred to in shorthand as "retro Diels Alder reaction."

Retro Diels-Alder reaction

The Retro Diels-Alder coupling chemistry generally involves a pair of reactants that couple to form an unstable intermediate, which intermediate eliminates a small molecule (depending on the starting compounds this may be e.g. N_2, CO_2 , RCN), as the sole by-product through a retro Diels-Alder reaction to form the retro Diels-Alder adduct. The paired reactants comprise, as one reactant (i.e. one Bio-orthogonal Reactive Group), a suitable diene, such as a derivative of tetrazine, e.g. an electron-deficient tetrazine and, as the other reactant (i.e. the other Bio-orthogonal Reactive Group), a suitable dienophile, such as a strained cyclooctene (TCO).

The exceptionally fast reaction of e.g. electron-deficient (substituted) tetrazines with a TCO moiety results in a ligation intermediate that rearranges to a dihydropyridazine retro Diels-Alder adduct by eliminating N_2 as the sole by-product in a [4+2] Retro Diels-Alder cycloaddition. In aqueous environment, the inititally formed 4,5-dihydropyridazine product may tautomerize to a 1,4-dihydropyridazine product.

The two reactive species are abiotic and do not undergo fast metabolism or side reactions in vivo. They are bio-orthogonal, e.g. they selectively react with each other in physiologic media. Thus, the compounds and the method of the invention can be used in a living organism. Moreover, the reactive groups are relatively small and can be introduced in biological samples or living organisms without significantly altering the size of biomolecules therein. References on the Inverse electron demand Diels Alder reaction, and the behavior of the pair of reactive species include: Thalhammer, F; Wallfahrer, U; Sauer, J, Tetrahedron Letters, 1990, 31 (47), 6851-6854; Wijnen, JW; Zavarise, S; Engberts, JBFN, Journal Of Organic Chemistry, 1996, 61, 2001-2005; Blackman, ML; Royzen, M; Fox, JM, Journal Of The American Chemical Society, 2008, 130 (41), 13518-19), R. Rossin, P. Renart Verkerk, Sandra M. van den Bosch, R. C. M. Vulders, l. Verel, J. Lub, M. S. Robillard, Angew Chem Int Ed 2010, 49, 3375, N. K. Devaraj, R. Upadhyay, J. B. Haun, S. A. Hilderbrand, R. Weissleder, Angew Chem Int Ed 2009, 48, 7013, and Devaraj et al., Angew.Chem.Int.Ed., 2009, 48, 1-5.

It will be understood that, in a broad sense, according to the invention the aforementioned retro Diels-Alder coupling and subsequent drug activation chemistry can be applied to basically any pair of molecules, groups, or moieties that are capable of being used in Prodrug therapy. I.e. one of such a pair will comprise a drug linked to a dienophile (the Trigger). The other one will be a complementary diene for use in reaction with said dienophile.

Trigger

The Prodrug comprises a Drug denoted as D^D linked, directly or indirectly, to a Trigger moiety denoted as T^R, wherein the Trigger moiety is a dienophile. The dienophile, in a broad sense, is an eight-membered non-aromatic cyclic alkenylene moiety (preferably a cyclooctene moiety, and more preferably a *trans*-cyclooctene moiety).

In this invention, the release of the drug or drugs is caused by an intramolecular cyclization/elimination reaction within the part of the Retro Diels-Alder adduct that originates from the TCO dienophile. A nucleophilic site present on the TCO (i.e. the dienophile, particularly from the Y^D group in this Trigger, *vide supra*) reacts with an electrophilic site on the same TCO (particularly from the X^D group in this Trigger, *vide supra*) after this TCO reacts with the Activator to form an rDA adduct. The part of the rDA adduct that originates from the TCO, i.e. the eight membered ring of the rDA adduct, has a different conformation and has an increased conformational freedom compared to the eight membered ring in the TCO prior to the rDA reaction, allowing the nucleophilic reaction to occur, thereby releasing the drug as a leaving group. The intramolecular cyclization/elimination reaction takes place, as the nucleophilic site and the electrophilic site have been brought together in close proximity within the Retro Diels-Alder adduct, and produce a favorable structure with a low strain. Additionally, the formation of the cyclic structure may also be a driving force for the intramolecular reaction to take place, and thus may also contribute to an effective release of the leaving group, i.e. release of the drug. Reaction between the nucleophilic site and the electrophilic site does not take place or is relatively inefficient prior to the Retro Diels-Alder reaction, as both sites are positioned unfavorably for such a reaction, due to the relatively rigid, conformationally restrained TCO ring. The Prodrug itself is relatively stable as such and elimination is favored only after the Activator and the Prodrug have reacted and have been assembled in a retro Diels-Alder adduct that is subject to intramolecular reaction. In a preferred embodiment the TCO ring is in the crown conformation. The example below illustrates the release mechanism pertaining to this invention.

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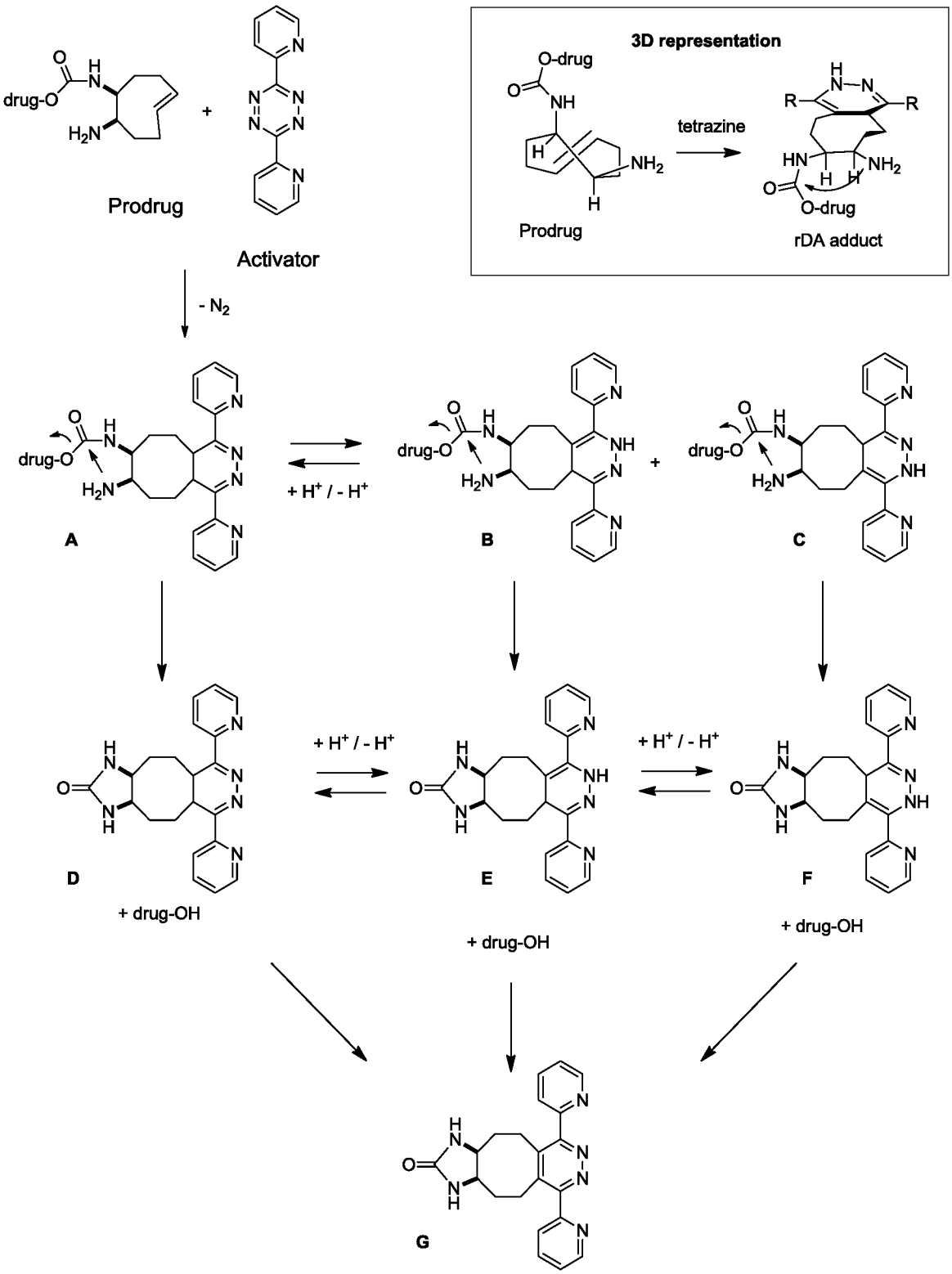

The above example illustrates how the intramolecular cyclization/elimination reaction within the retro Diels-Alder adduct can result in release of a drug species. The rDA reaction produces A, which may tautomerize to product B and C. Structures B and C may also tautomerize to one another (not shown). rDA products A, B, and C may intramolecularly cyclize, releasing the bound drug, and affording structures D, E, and F, which optionally may

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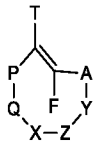
oxidise to form product G. As the tautomerization of A into B and C in water is very fast (in the order of seconds) it is the inventors' belief, that drug release occurs predominantly from structures B and C. It may also be possible that the nucleophilic site assists in expelling the drug species by a nucleophilic attack on the electrophilic site with subsequent drug release, but without actually forming a (stable) cyclic structure. In this case, no ring structure is formed and the nucleophilic site remains intact, for example because the ring structure is shortlived and unstable and breaks down with reformation of the nucleophilic site. In any case, and in whatever way the process is viewed, the drug species (here the alcohol 'drug-OH') is effectively expelled from the retro Diels-Alder adduct, while it does not get expelled from the Prodrug alone.

Without wishing to be bound by theory, the above example illustrates how the conformational restriction and the resulting unfavorable positioning of the nucleophilic and electrophilic site in the TCO trigger is relieved following rDA adduct formation, leading to an elimination/cyclization reaction and release of the drug.

With respect to the nucleophilic site on the TCO, one has to consider that the site must be able to act as a nucleophile under conditions that may exist inside the (human) body, so for example at physiological conditions where the pH = ca. 7.4, or for example at conditions that prevail in malignant tissue where pH-values may be lower than 7.4. Preferred nucleophiles are amine, thiol or alcohol groups, as these are generally most nucleophilic in nature and therefore most effective.

In this invention, the TCO satisfies the following formula (1a):



(1a)

In Embodiment 1, one of the bonds PQ, QP, QX, XQ, XZ, ZX, ZY, YZ, YA, AY consists of $-CR^aX^D-CR^aY^D-$, the remaining groups (from A,Y,Z,X,Q,P) being independently from each other CR^a_2, S, O, SiR^b_2, such that P and A are CR^a_2, and no adjacent pairs of atoms are present selected from the group consisting of O-O, O-S, and S-S, and such that Si, if present, is adjacent to CR^a_2 or O.

X^D is O-C(O)-$(L^D)_n$-(D^D), S-C(O)-$(L^D)_n$-(D^D), O-C(S)-$(L^D)_n$-(D^D), S-C(S)-$(L^D)_n$-(D^D), NR^c-C(O)-$(L^D)_n$-(D^D), NR^c-C(S)-$(L^D)_n$-(D^D), and then Y^D is NHR^c, OH, SH; or

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X^D is C(O)-$(L^D)_n$-(D^D), C(S)-$(L^D)_n$-(D^D); and then Y^D is $CR^c_2NHR^c$, CR^c_2OH, CR^c_2SH, NH-NH_2, O-NH_2, NH-OH.

Preferably X^D is NR^c-C(O)-$(L^D)_n$-(D^D), and Y^D is NHR^c.

In this Embodiment 1, the X^D and Y^D groups may be positioned *cis* or *trans* relative to each other, where depending on the positions on the TCO, *cis* or *trans* are preferred: if PQ, QP, AY or YA is -CR^aX^D-CR^aY^D-, then X^D and Y^D are preferably positioned *trans* relative to each other; if ZX or XZ is -CR^aX^D-CR^aY^D -, then X^D and Y^D are preferably positioned *cis* relative to each other.

In Embodiment 2, A is CR^aX^D and Z is CR^aY^D, or Z is CR^aX^D and A is CR^aY^D, or P is CR^aX^D and X is CR^aY^D, or X is CR^aX^D and P is CR^aY^D, such that X^D and Y^D are positioned in a *trans* conformation with respect to one another; the remaining groups (from A,Y,Z,X,Q,P) being independently from each other CR^a_2, S, O, SiR^b_2, such that P and A are CR^a_2, and no adjacent pairs of atoms are present selected from the group consisting of O-O, O-S, and S-S, and such that Si, if present, is adjacent to CR^a_2 or O; X^D is O-C(O)-$(L^D)_n$-(D^D), S-C(O)-$(L^D)_n$-(D^D), O-C(S)-$(L^D)_n$-(D^D), S-C(S)-$(L^D)_n$-(D^D), NR^c-C(O)-$(L^D)_n$-(D^D), NR^c-C(S)-$(L^D)_n$-(D^D), and then Y^D is NHR^c, OH, SH, $CR^c_2NHR^c$, CR^c_2OH, CR^c_2SH, NH-NH_2, O-NH_2, NH-OH; or X^D is CR^c_2-O-C(O)-$(L^D)_n$-(D^D), CR^c_2-S-C(O)-$(L^D)_n$-(D^D), CR^c_2-O-C(S)-$(L^D)_n$-(D^D), CR^c_2-S-C(S)-$(L^D)_n$-(D^D), CR^c_2-NR^c-C(O)-$(L^D)_n$-(D^D), CR^c_2-NR^c-C(S)-$(L^D)_n$-(D^D); and then Y^D is NHR^c, OH, SH; or X^D is C(O)-$(L^D)_n$-(D^D), C(S)-$(L^D)_n$-(D^D); and then Y^D is $CR^c_2NHR^c$, CR^c_2OH, CR^c_2SH, NH-NH_2, O-NH_2, NH-OH.

Preferably X^D is NR^c-C(O)-$(L^D)_n$-(D^D), and Y^D is NHR^c.

In Embodiment 3, A is CR^aY^D and one of P, Q, X, Z is CR^aX^D, or P is CR^aY^D and one of A, Y, Z, X is CR^aX^D, or Y is CR^aY^D and X or P is CR^aX^D, or Q is CR^aY^D and Z or A is CR^aX^D, or either Z or X is CR^aY^D and A or P is CR^aX^D, such that X^D and Y^D are positioned in a *trans* conformation with respect to one another; the remaining groups (from A,Y,Z,X,Q,P) being independently from each other CR^a_2, S, O, SiR^b_2, such that P and A are CR^a_2, and no adjacent pairs of atoms are present selected from the group consisting of O-O, O-S, and S-S, and such that Si, if present, is adjacent to CR^a_2 or O.

X^D is $(O\text{-}C(O))_p$-$(L^D)_n$-(D^D), S-C(O)-$(L^D)_n$-(D^D), O-C(S)-$(L^D)_n$-(D^D), S-C(S)-$(L^D)_n$-(D^D); Y^D is $CR^c_2NHR^c$, CR^c_2OH, CR^c_2SH, NH-NH_2, O-NH_2, NH-OH; p = 0 or 1.

Preferably X^D is $(O\text{-}C(O))_p$-$(L^D)_n$-(D^D), with p=1, and Y^D is $CR^c_2NHR^c$.

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In Embodiment 4, P is CR^aY^D and Y is CR^aX^D, or A is CR^aY^D and Q is CR^aX^D, or Q is CR^aY^D and A is CR^aX^D, or Y is CR^aY^D and P is CR^aX^D, such that X^D and Y^D are positioned in a *trans* conformation with respect to one another; the remaining groups (from A,Y,Z,X,Q,P) being independently from each other CR^a_2, S, O, SiR^b_2, such that P and A are CR^a_2, and no adjacent pairs of atoms are present selected from the group consisting of O-O, O-S, and S-S, and such that Si, if present, is adjacent to CR^a_2 or O.

X^D is $(O\text{-}C(O))_p\text{-}(L^D)_n\text{-}(D^D)$, $S\text{-}C(O)\text{-}(L^D)_n\text{-}(D^D)$, $O\text{-}C(S)\text{-}(L^D)_n\text{-}(D^D)$, $S\text{-}C(S)\text{-}(L^D)_n\text{-}(D^D)$; Y^D is NHR^c, OH, SH; p = 0 or 1.

Preferably X^D is $(O\text{-}C(O))_p\text{-}(L^D)_n\text{-}(D^D)$, with p=1, and Y^D is NHR^c.

In Embodiment 5, Y is Y^D and P is CR^aX^D, or Q is Y^D and A is CR^aX^D; the remaining groups (from A,Y,Z,X,Q,P) being independently from each other CR^a_2, S, O, SiR^b_2, such that P and A are CR^a_2, and no adjacent pairs of atoms are present selected from the group consisting of O-O, O-S, and S-S, and such that Si, if present, is adjacent to CR^a_2 or O.

X^D is $(O\text{-}C(O))_p\text{-}(L^D)_n\text{-}(D^D)$, $S\text{-}C(O)\text{-}(L^D)_n\text{-}(D^D)$, $O\text{-}C(S)\text{-}(L^D)_n\text{-}(D^D)$, $S\text{-}C(S)\text{-}(L^D)_n\text{-}(D^D)$, $NR^c\text{-}C(O)\text{-}(L^D)_n\text{-}(D^D)$, $NR^c\text{-}C(S)\text{-}(L^D)_n\text{-}(D^D)$, $C(O)\text{-}(L^D)_n\text{-}(D^D)$, $C(S)\text{-}(L^D)_n\text{-}(D^D)$; Y^D is NH; p = 0 or 1.

Preferably X^D is $NR^c\text{-}C(O)\text{-}(L^D)_n\text{-}(D^D)$ or $(O\text{-}C(O))_p\text{-}(L^D)_n\text{-}(D^D)$, with p = 0 or 1.

In Embodiment 6, Y is Y^D and P or Q is X^D, or Q is Y^D and A or Y is X^D; the remaining groups (from A,Y,Z,X,Q,P) being independently from each other CR^a_2, S, O, SiR^b_2, such that P and A are CR^a_2, and no adjacent pairs of atoms are present selected from the group consisting of O-O, O-S, and S-S, and such that Si, if present, is adjacent to CR^a_2 or O.

X^D is $N\text{-}C(O)\text{-}(L^D)_n\text{-}(D^D)$, $N\text{-}C(S)\text{-}(L^D)_n\text{-}(D^D)$; Y^D is NH;

Preferably X^D is $N\text{-}C(O)\text{-}(L^D)_n\text{-}(D^D)$

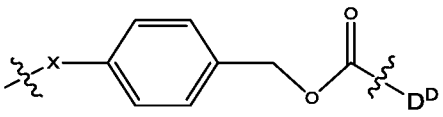
T, F each independently denotes H, or a substituent selected from the group consisting of alkyl, F, Cl, Br, or I. $(L^D)_n$ is an optional linker with n= 0 or 1, preferably linked to T^R via S, N, NH, or O, wherein these atoms are part of the linker, which may consist of multiple units arranged linearly and/or branched. D^D is one or more therapeutic moieties or drugs, preferably linked via S, N, NH, or O, wherein these atoms are part of the therapeutic moiety.

In a preferred embodiment, the TCO of formula (1a) is an all-carbon ring. In another preferred embodiment, the TCO of formula (1a) is a heterocyclic carbon ring, having of one or two oxygen atoms in the ring, and preferably a single oxygen atom.

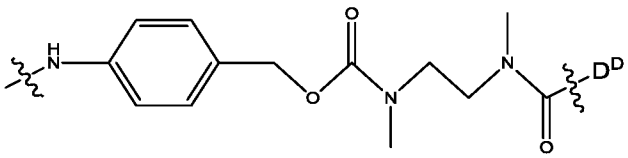
It is preferred that when D^D is bound to T^R or L^D via NH, this NH is a primary amine ($-NH_2$) residue from D^D, and when D^D is bound via N, this N is a secondary amine (-NH-) residue from D^D. Similarly, it is preferred that when D^D is bound via O or S, said O or S are, respectively, a hydroxyl (-OH) residue or a sulfhydryl (-SH) residue from D^D.
It is further preferred that said S, N, NH, or O moieties comprised in D^D are bound to an aliphatic or aromatic carbon of D^D.

It is preferred that when L^D is bound to T^R via NH, this NH is a primary amine ($-NH_2$) residue from L^D, and when L^D is bound via N, this N is a secondary amine (-NH-) residue from L^D. Similarly, it is preferred that when L^D is bound via O or S, said O or S are, respectively, a hydroxyl (-OH) residue or a sulfhydryl (-SH) residue from L^D.
It is further preferred that said S, N, NH, or O moieties comprised in L^D are bound to an aliphatic or aromatic carbon of L^D.

Where reference is made in the invention to a linker L^D this can be self-immolative or not, or a combination thereof, and which may consist of multiple self-immolative units. By way of further clarification, if p=0 and n=0 the drug species D^D directly constitutes the leaving group of the elimination reaction, and if p=0 and n=1, the self-immolative linker constitutes the leaving group of the elimination. The position and ways of attachment of linkers L^D and drugs D^D are known to the skilled person (see for example Papot et al, *Anti-Cancer Agents in Medicinal Chemistry*, **2008**, 8, 618-637). Nevertheless, typical but non-limiting examples of self-immolative linkers L^D are benzyl-derivatives, such as those drawn below. On the right, an example of a self-immolative linker with multiple units is shown; this linker will degrade not only into CO_2 and one unit of 4-aminobenzyl alcohol, but also into one 1,3-dimethylimidazolidin-2-one unit.

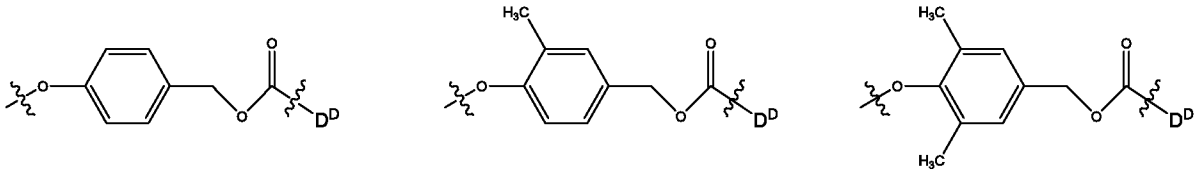


X = O or S or NH or NR with R = alkyl or aryl



By substituting the benzyl groups of aforementioned self-immolative linkers L^D, preferably on the 2- and/or 6-position, it may be possible to tune the rate of release of the

drug species D^D, caused by either steric and/or electronic effects on the intramolecular cyclization/elimination reaction. Synthetic procedures to prepare such substituted benzyl-derivatives are known to the skilled person (see for example Greenwald et al, *J. Med. Chem.*, **1999**, 42, 3657-3667 and Thornthwaite et al, *Polym. Chem.*, **2011**, 2, 773-790). Some examples of substituted benzyl-derivatives with different release rates are drawn below.



Each R^a as above-indicated can independently be H, alkyl, aryl, OR', SR', S(=O)R''', $S(=O)_2$R''', $S(=O)_2$NR'R'', Si-R''', Si-O-R''', OC(=O)R''', SC(=O)R''', OC(=S)R''', SC(=S)R''', F, Cl, Br, I, N_3, SO_2H, SO_3H, SO_4H, PO_3H, PO_4H, NO, NO_2, CN, OCN, SCN, NCO, NCS, CF_3, CF_2-R', NR'R", C(=O)R', C(=S)R', C(=O)O-R', C(=S)O-R', C(=O)S-R', C(=S)S-R', C(=O)NR'R", C(=S)NR'R'', NR'C(=O)-R''', NR'C(=S)-R''', NR'C(=O)O-R''', NR'C(=S)O-R''', NR'C(=O)S-R''', NR'C(=S)S-R''', OC(=O)NR'-R''', SC(=O)NR'-R''', OC(=S)NR'-R''', SC(=S)NR'-R''', NR'C(=O)NR"-R'', NR'C(=S)NR"-R'', CR'NR'', with each R' and each R'' independently being H, aryl or alkyl and R''' independently being aryl or alkyl;

Each R^b as above indicated is independently selected from the group consisting of H, alkyl, aryl, O-alkyl, O-aryl, OH;

Each R^c as above indicated is independently selected from H, C_{1-6} alkyl and C_{1-6} aryl;

wherein two or more $R^{a,b,c}$ moieties together may form a ring;

Preferably, each R^a is independently selected from the group consisting of H, alkyl, O-alkyl, O-aryl, OH, C(=O)NR'R", NR'C(=O)-R''', with R' and R" each independently being H, aryl or alkyl, and with R''' independently being alkyl or aryl.

In all of the above embodiments, optionally one of A, P, Q, Y, X, and Z, or the substituents, or the self-immolative linker L^D, or the drug D^D, is bound, optionally via a spacer or spacers S^P, to one or more targeting agents T^T or masking moieties M^M.

The synthesis of TCO's as described above is well available to the skilled person. This expressly also holds for TCO's having one or more heteroatoms in the strained cycloalkene rings. References in this regard include Cere *et al. Journal of Organic Chemistry* 1980, *45*, 261 and Prevost et al. *Journal of the American Chemical Society* 2009, *131*, 14182.

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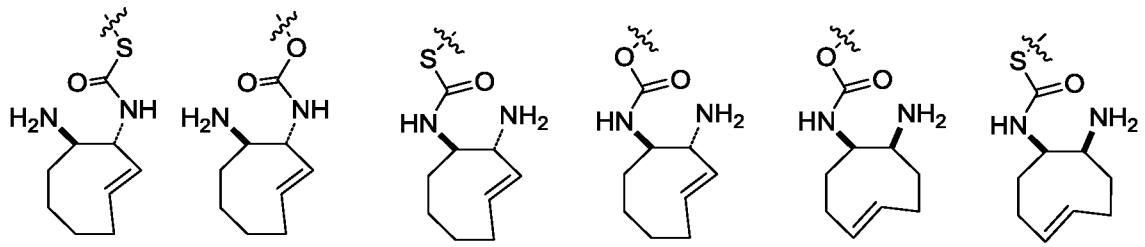
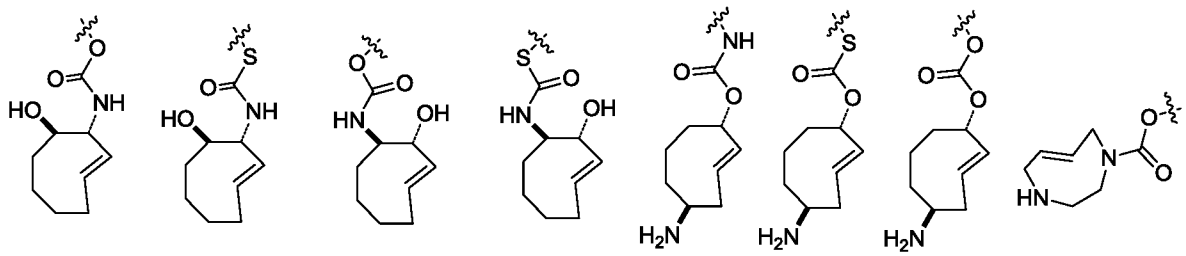
In a further preferred embodiment, the dienophile is a compound selected from the following structures:



〰 = rest of attached D^D, L^D-D^D, optionally comprising T^T or S^P-T^T or M^M or S^P-M^M

In alternative embodiments, the dienophile is a compound selected from the following structures:



〰 = rest of attached D^D, L^D-D^D, optionally comprising T^T or S^P-T^T or M^M or S^P-M^M

Use of TCO as a carrier

The invention also pertains to the use of a *trans*-cyclooctene satisfying formula (1a), in all its embodiments, as a carrier for a therapeutic compound. The *trans*-cyclooctene is to be read as a TCO in a broad sense, as discussed above, preferably an all-carbon ring or including one or two hetero-atoms. A therapeutic compound is a drug or other compound or moiety intended to have therapeutic application. The use of TCO as a carrier according to this aspect of the invention does not relate to the therapeutic activity of the therapeutic compound. In fact, also if the therapeutic compound is a drug substance intended to be developed as a drug, many of which will fail in practice, the application of TCO as a carrier still is useful in testing the drug. In this sense, the TCO in its capacity of a carrier is to be regarded in the same manner as a pharmaceutical excipient, serving as a carrier when introducing a drug into a subject.

The use of a TCO as a carrier has the benefit that it enables the administration, to a subject, of a drug carried by a moiety that is open to a bioorthogonal reaction, with a diene, particularly a tetrazine. This provides a powerful tool not only to affect the fate of the drug carried into the body, but also to follow its fate (e.g. by allowing a labeled diene to react

with it), or to change its fate (e.g. by allowing pK modifying agents to bind with it). This is all based on the possibility to let a diene react with the TCO in the above-discussed rDA reaction. The carrier is preferably reacted with an Activator as discussed below, so as to provoke the release of the therapeutic compound from the TCO, as amply discussed herein.

Activator

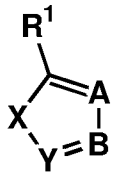
The Activator comprises a Bio-orthogonal Reactive Group, wherein this Bio-orthogonal Reactive Group of the Activator is a diene. This diene reacts with the other Bio-orthogonal Reactive Group, the Trigger, and that is a dienophile (*vide supra*). The diene of the Activator is selected so as to be capable of reacting with the dienophile of the Trigger by undergoing a Diels-Alder cycloaddition followed by a retro Diels-Alder reaction, giving the Retro Diels-Alder adduct. This intermediate adduct then releases the drug or drugs, where this drug release can be caused by various circumstances or conditions that relate to the specific molecular structure of the retro Diels-Alder adduct.

Dienes

The person skilled in the art is aware of the wealth of dienes that are reactive in the Retro Diels-Alder reaction. The diene comprised in the Activator can be part of a ring structure that comprises a third double bond, such as a tetrazine (which is a preferred Activator according to the invention).

Generally, the Activator is a molecule comprising a heterocyclic moiety comprising at least 2 conjugated double bonds.

Preferred dienes are given below, with reference to formulae (2)-(4).



(2)

In formula (2) R^1 is selected from the group consisting of H, alkyl, aryl, CF_3, CF_2-R', OR', SR', C(=O)R', C(=S)R', C(=O)O-R', C(=O)S-R', C(=S)O-R', C(=S)S-R'', C(=O)NR'R'', C(=S)NR'R'', NR'R'', NR'C(=O)R'', NR'C(=S)R'', NR'C(=O)OR'', NR'C(=S)OR'', NR'C(=O)SR'', NR'C(=S)SR'', NR'C(=O)NR''R'', NR'C(=S)NR''R'' with each R' and each R'' independently being H, aryl or alkyl; A and B each independently are selected from the group consisting of alkyl-substituted carbon, aryl substituted carbon,

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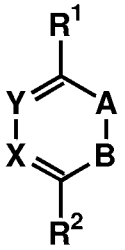
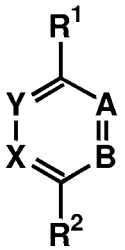
nitrogen, N^+O^-, N^+R with R being alkyl, with the proviso that A and B are not both carbon; X is selected from the group consisting of O, N-alkyl, and C=O, and Y is CR with R being selected from the group consisting of H, alkyl, aryl, C(=O)OR', C(=O)SR', C(=S)OR', C(=S)SR', C(=O)NR'R'' with R' and R'' each independently being H, aryl or alkyl.



(3)

A diene particularly suitable as a reaction partner for cyclooctene is given in formula (3), wherein R^1 and R^2 each independently are selected from the group consisting of H, alkyl, aryl, CF_3, CF_2-R', NO_2, OR', SR', C(=O)R', C(=S)R', OC(=O)R''', SC(=O)R''', OC(=S)R''', SC(=S)R''', S(=O)R', $S(=O)_2$R''', $S(=O)_2$NR'R'', C(=O)O-R', C(=O)S-R', C(=S)O-R', C(=S)S-R', C(=O)NR'R'', C(=S)NR'R'', NR'R'', NR'C(=O)R'', NR'C(=S)R'', NR'C(=O)OR'', NR'C(=S)OR'', NR'C(=O)SR'', NR'C(=S)SR'', OC(=O)NR'R'', SC(=O)NR'R'', OC(=S)NR'R'', SC(=S)NR'R'', NR'C(=O)NR''R'', NR'C(=S)NR''R'' with each R' and each R'' independently being H, aryl or alkyl, and R''' independently being aryl or alkyl; A is selected from the group consisting of N-alkyl, N-aryl, C=O, and CN-alkyl; B is O or S; X is selected from the group consisting of N, CH, C-alkyl, C-aryl, CC(=O)R', CC(=S)R', CS(=O)R', $CS(=O)_2$R''', CC(=O)O-R', CC(=O)S-R', CC(=S)O-R', CC(=S)S-R', CC(=O)NR'R'', CC(=S)NR'R'', R' and R'' each independently being H, aryl or alkyl and R''' independently being aryl or alkyl; Y is selected from the group consisting of CH, C-alkyl, C-aryl, N, and N^+O^-.



(4)

Another diene particularly suitable as a reaction partner for cyclooctene is diene (4), wherein R^1 and R^2 each independently are selected from the group consisting of H, alkyl, aryl, CF_3, CF_2-R', NO, NO_2, OR', SR', CN, C(=O)R', C(=S)R', OC(=O)R''', SC(=O)R''', OC(=S)R''', SC(=S)R''', S(=O)R', $S(=O)_2$R''', $S(=O)_2$OR', PO_3R'R",

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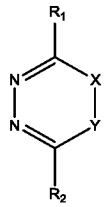
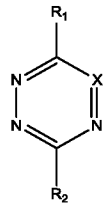
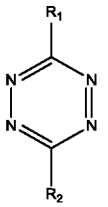
$S(=O)_2NR'R''$, C(=O)O-R', C(=O)S-R', C(=S)O-R', C(=S)S-R', C(=O)NR'R'', C(=S)NR'R'', NR'R'', NR'C(=O)R'', NR'C(=S)R'', NR'C(=O)OR'', NR'C(=S)OR'', NR'C(=O)SR'', NR'C(=S)SR'', OC(=O)NR'R'', SC(=O)NR'R'', OC(=S)NR'R'', SC(=S)NR'R'', NR'C(=O)NR''R'', NR'C(=S)NR''R'' with each R' and each R'' independently being H, aryl or alkyl, and R''' independently being aryl or alkyl; A is selected from the group consisting of N, C-alkyl, C-aryl, and N^+O^-; B is N; X is selected from the group consisting of N, CH, C-alkyl, C-aryl, CC(=O)R', CC(=S)R', CS(=O)R', $CS(=O)_2R'''$, CC(=O)O-R', CC(=O)S-R', CC(=S)O-R', CC(=S)S-R', CC(=O)NR'R'', CC(=S)NR'R'', R' and R'' each independently being H, aryl or alkyl and R''' independently being aryl or alkyl; Y is selected from the group consisting of CH, C-alkyl, C-aryl, N, and N^+O^-.



(5)



(6)



(7)

According to the invention, particularly useful dienes are 1,2-diazine, 1,2,4-triazine and 1,2,4,5-tetrazine derivatives, as given in formulas (5), (6) and (7), respectively.

The 1,2-diazine is given in (5), wherein R^1 and R^2 each independently are selected from the group consisting of H, alkyl, aryl, CF_3, CF_2-R', NO_2, OR', SR', C(=O)R', C(=S)R', OC(=O)R''', SC(=O)R''', OC(=S)R''', SC(=S)R''', S(=O)R', $S(=O)_2R'''$, $S(=O)_2NR'R''$, C(=O)O-R', C(=O)S-R', C(=S)O-R', C(=S)S-R', C(=O)NR'R'', C(=S)NR'R'', NR'R'', NR'C(=O)R'', NR'C(=S)R'', NR'C(=O)OR'', NR'C(=S)OR'', NR'C(=O)SR'', NR'C(=S)SR'', OC(=O)NR'R'', SC(=O)NR'R'', OC(=S)NR'R'', SC(=S)NR'R'', NR'C(=O)NR''R'', NR'C(=S)NR''R'' with each R' and each R'' independently being H, aryl or alkyl, and R''' independently being aryl or alkyl; X and Y each independently are selected from the group consisting of O, N-alkyl, N-aryl, C=O, CN-alkyl, CH, C-alkyl, C-aryl, CC(=O)R', CC(=S)R', CS(=O)R', $CS(=O)_2R'''$, CC(=O)O-R', CC(=O)S-R', CC(=S)O-R', CC(=S)S-R', CC(=O)NR'R'', CC(=S)NR'R'', with R' and R'' each independently being H, aryl or alkyl and R''' independently being aryl or alkyl, where X-Y may be a single or a double bond, and where X and Y may be connected in a second ring structure apart from the 6-membered diazine. Preferably, X-Y represents an ester group (X = O and Y = C=O; X-Y is a single bond) or X-Y represents a cycloalkane group (X = CR' and Y = CR''; X-Y is a single bond; R' and R'' are connected), preferably a cyclopropane

ring, so that R' and R'' are connected to each other at the first carbon atom outside the 1,2-diazine ring.

The 1,2,4-triazine is given in (6), wherein R^1 and R^2 each independently are selected from the group consisting of H, alkyl, aryl, CF_3, CF_2-R', NO_2, OR', SR', C(=O)R', C(=S)R', OC(=O)R''', SC(=O)R''', OC(=S)R''', SC(=S)R''', S(=O)R', $S(=O)_2$R''', $S(=O)_2$NR'R'', C(=O)O-R', C(=O)S-R', C(=S)O-R', C(=S)S-R', C(=O)NR'R'', C(=S)NR'R'', NR'R'', NR'C(=O)R'', NR'C(=S)R'', NR'C(=O)OR'', NR'C(=S)OR'', NR'C(=O)SR'', NR'C(=S)SR'', OC(=O)NR'R'', SC(=O)NR'R'', OC(=S)NR'R'', SC(=S)NR'R'', NR'C(=O)NR''R'', NR'C(=S)NR''R'' with each R' and each R'' independently being H, aryl or alkyl, and R''' independently being aryl or alkyl; X is selected from the group consisting of CH, C-alkyl, C-aryl, CC(=O)R', CC(=S)R', CS(=O)R', $CS(=O)_2$R''', CC(=O)O-R', CC(=O)S-R', CC(=S)O-R', CC(=S)S-R', CC(=O)NR'R'', CC(=S)NR'R'', R' and R'' each independently being H, aryl or alkyl and R''' independently being aryl or alkyl.

The 1,2,4,5-tetrazine is given in (7), wherein R^1 and R^2 each independently are selected from the group consisting of H, alkyl, aryl, CF_3, CF_2-R', NO, NO_2, OR', SR', CN, C(=O)R', C(=S)R', OC(=O)R''', SC(=O)R''', OC(=S)R''', SC(=S)R''', S(=O)R', $S(=O)_2$R''', $S(=O)_2$OR', PO_3R'R", $S(=O)_2$NR'R'', C(=O)O-R', C(=O)S-R', C(=S)O-R', C(=S)S-R', C(=O)NR'R'', C(=S)NR'R'', NR'R'', NR'C(=O)R'', NR'C(=S)R'', NR'C(=O)OR'', NR'C(=S)OR'', NR'C(=O)SR'', NR'C(=S)SR'', OC(=O)NR'R'', SC(=O)NR'R'', OC(=S)NR'R'', SC(=S)NR'R'', NR'C(=O)NR''R'', NR'C(=S)NR''R'' with each R' and each R'' independently being H, aryl or alkyl, and R''' independently being aryl or alkyl.

Electron-deficient 1,2-diazines (5), 1,2,4-triazines (6) or 1,2,4,5-tetrazines (7) are especially interesting as such dienes are generally more reactive towards dienophiles. Di-tri- or tetra-azines are electron deficient when they are substituted with groups or moieties that do not generally hold as electron-donating, or with groups that are electron-withdrawing. For example, R^1 and/or R^2 may denote a substituent selected from the group consisting of H, alkyl, NO_2, F, Cl, CF_3, CN, COOR, CONHR, $CONR_2$, COR, SO_2R, SO_2OR, SO_2NR_2, PO_3R_2, NO, 2-pyridyl, 3-pyridyl, 4-pyridyl, 2,6-pyrimidyl, 3,5-pyrimidyl, 2,4-pyrimidyl, 2,4 imidazyl, 2,5 imidazyl or phenyl, optionally substituted with one or more electron-withdrawing groups such as NO_2, F, Cl, CF_3, CN, COOR, CONHR, CONR, COR, SO_2R, SO_2OR, SO_2NR_2, PO_3R_2, NO, Ar, wherein R is H or C_1-C_6 alkyl, and Ar stands for an aromatic group, particularly phenyl, pyridyl, or naphthyl.

The 1,2,4,5-tetrazines of formula (7) are most preferred as Activator dienes, as these molecules are generally most reactive in retro Diels-Alder reactions with dienophiles, such as the preferred TCO dienophiles, even when the R^1 and/or R^2 groups are not necessarily electron withdrawing, and even when R^1 and/or R^2 are in fact electron donating. Electron donating groups are for example OH, OR', SH, SR', NH_2, NHR', NR'R'', NHC(=O)R'', NR'C(=O)R'', NHC(=S)R'', NR'C(=S)R'', $NHSO_2R''$, $NR'SO_2R''$ with R' and R'' each independently being alkyl or aryl groups. Examples of other electron donating groups are phenyl groups with attached to them one or more of the electron donating groups as mentioned in the list above, especially when substituted in the 2-, 4- and/or 6-position(s) of the phenyl group.

According to the invention, 1,2,4,5-tetrazines with two electron withdrawing residues, or those with one electron withdrawing residue and one residue that is neither electron withdrawing nor donating, are called electron deficient. In a similar way, 1,2,4,5-tetrazines with two electron donating residues, or those with one electron donating residue and one residue that is neither electron withdrawing nor donating, are called electron sufficient. 1,2,4,5-Tetrazines with two residues that are both neither electron withdrawing nor donating, or those that have one electron withdrawing residue and one electron donating residue, are neither electron deficient nor electron sufficient.

The 1,2,4,5-tetrazines can be asymmetric or symmetric in nature, i.e. the R^1 and R^2 groups in formula (7) may be different groups or may be identical groups, respectively. Symmetric 1,2,4,5-tetrazines are more convenient as these Activators are more easily accessible via synthetic procedures.

We have tested several 1,2,4,5-tetrazines with respect to their ability as Activator to release a model drug compound (e.g. phenol) from a Prodrug via an elimination process, and we have found that tetrazines that are electron deficient, electron sufficient or neither electron deficient nor electron sufficient are capable to induce the drug release. Furthermore, both symmetric as well as asymmetric tetrazines were effective.

Electron deficient 1,2,4,5-tetrazines and 1,2,4,5-tetrazines that are neither electron deficient nor electron sufficient are generally more reactive in retro Diels-Alder reactions with dienophiles (such as TCOs), so these two classes of 1,2,4,5-tetrazines are preferred over electron sufficient 1,2,4,5-tetrazines, even though the latter are also capable of inducing drug release in Prodrugs.

Therefore, particularly useful tetrazine derivatives are electron-deficient tetrazines, i.e. tetrazines substituted with groups or moieties that do not generally hold as

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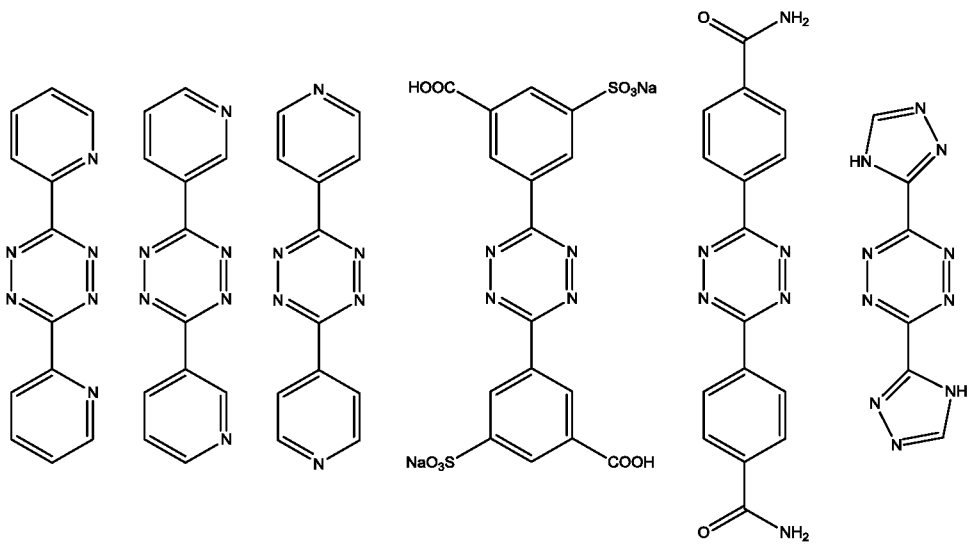
electron-donating, and preferably carrying electron-withdrawing substituents. With reference to formula (7), for electron-deficient tetrazines, R^1 and R^2 each independently denote a substituent selected from the group consisting of 2-pyridyl, 3, pyridyl, 4-pyridyl, 2,6-pyrimidyl, 3,5-pyrimidyl, 2,4-pyrimidyl , or phenyl, optionally substituted with one or more electron-withdrawing groups such as NO_2, F, Cl, CF_3, CN, COOH, COOR, $CONH_2$, CONHR, $CONR_2$, CHO, COR, SO_2R, SO_2OR, NO, Ar, wherein R is C_1-C_6 alkyl and Ar stands for an aromatic group, particularly phenyl, pyridyl, or naphthyl.

In the compounds according to each of the formulae (2)-(7), the R^1 and R^2 groups can further be provided with suitable linker or spacer moieties as discussed herein.

In the following paragraphs specific examples of 1,2,4,5-tetrazine Activators will be highlighted by defining the R^1 and R^2 residues in formula (7).

Symmetric electron deficient 1,2,4,5-tetrazines with electron withdrawing residues are for example those with $R^1 = R^2 =$ H, 2-pyridyl, 3-pyridyl, 4-pyridyl, 2,4-pyrimidyl, 2,6-pyrimidyl, 3,5-pyrimidyl, 2,3,4-triazyl or 2,3,5-triazyl. Other examples are those with $R^1 = R^2 =$ phenyl with COOH or COOMe carboxylate, or with CN nitrile, or with $CONH_2$, $CONHCH_3$ or $CON(CH_3)_2$ amide, or with SO_3H or SO_3Na sulfonate, or with SO_2NH_2, SO_2NHCH_3 or $SO_2N(CH_3)_2$ sulfonamide, or with PO_3H_2 or PO_3Na_2 phosphonate substituents in the 2-, 3- or 4- position of the phenyl group, or in the 3- and 5-positions, or in the 2- and 4-positions, or in the 2,- and 6-positions of the phenyl group. Other substitution patterns are also possible, including the use of different substituents, as long as the tetrazine remains symmetric. See below for some examples of these structures.



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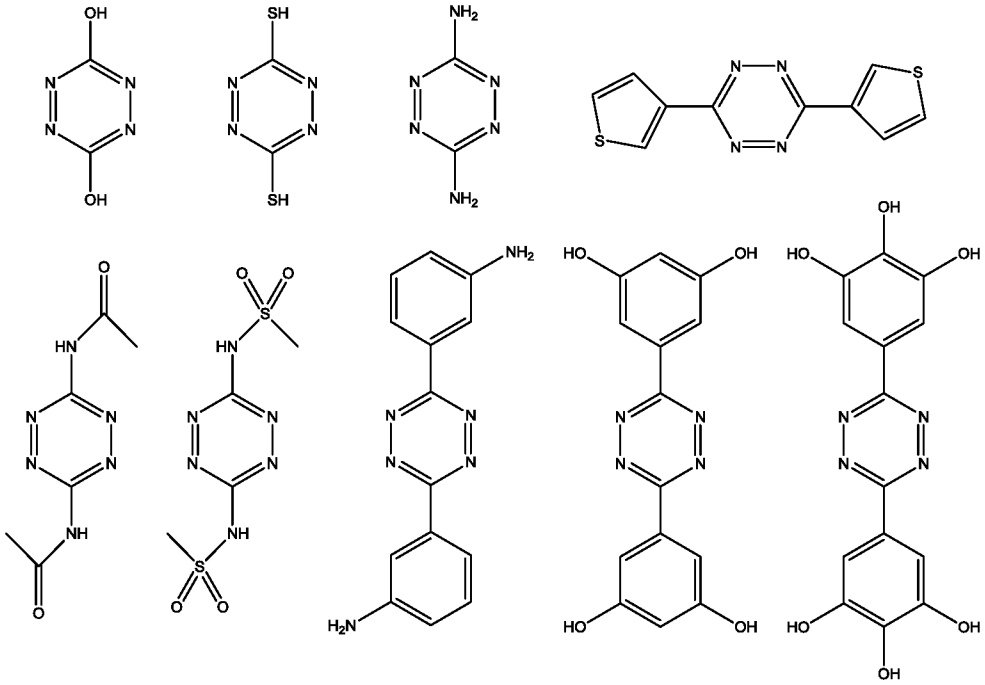
Symmetric electron sufficient 1,2,4,5-tetrazines with electron donating residues are for example those with $R^1 = R^2$ = OH, OR', SH, SR', NH_2, NHR', NR'_2, NH-CO-R', NH-SO-R', NH-SO_2-R', 2-pyrryl, 3-pyrryl, 2-thiophene, 3-thiophene, where R' represents a methyl, ethyl, phenyl or tolyl group. Other examples are those with $R^1 = R^2$ = phenyl with OH, OR', SH, SR', NH_2, NHR', NR'_2, NH-CO-R', NR"-CO-R', NH-SO-R' or NH-SO_2-R' substituent(s), where R' represents a methyl, ethyl, phenyl or tolyl group, where R" represents a methyl or ethyl group, and where the substitution is done on the 2- or 3- or 4- or 2- and 3- or 2- and 4- or 2- and 5- or 2- and 6- or 3- and 4- or 3- and 5- or 3-, 4- and 5-position(s). See below for some examples of these structures.



Symmetric 1,2,4,5-tetrazines with neither electron withdrawing nor electron donating residues are for example those with $R^1 = R^2$ = phenyl, methyl, ethyl, (iso)propyl, 2,4-imidazyl, 2,5-imidazyl, 2,3-pyrazyl or 3,4-pyrazyl. Other examples are those where $R^1 = R^2$ = a hetero(aromatic) cycle such as a oxazole, isoxazole, thiazole or oxazoline cycle. Other examples are those where $R^1 = R^2$ = a phenyl with one electron withdrawing substituent selected from COOH, COOMe, CN, $CONH_2$, $CONHCH_3$, $CON(CH_3)_2$, SO_3H, SO_3Na, SO_2NH_2, SO_2NHCH_3, $SO_2N(CH_3)_2$, PO_3H_2 or PO_3Na_2 and one electron donating subsituent selected from OH, OR', SH, SR', NH_2, NHR', NR'_2, NH-CO-R', NR"-CO-R', NH-SO-R' or NH-SO_2-R' substituent(s), where R' represents a methyl, ethyl, phenyl or tolyl group and where R" represents a methyl or ethyl group. Substitutions can be done on the 2- and 3-, 2- and 4-, 2,- and 5-, 2- and 6, 3- and 4-, and the 3- and 5-positions. Yet other examples are those where $R^1 = R^2$ = a pyridyl or pyrimidyl moiety with one electron donating subsituent

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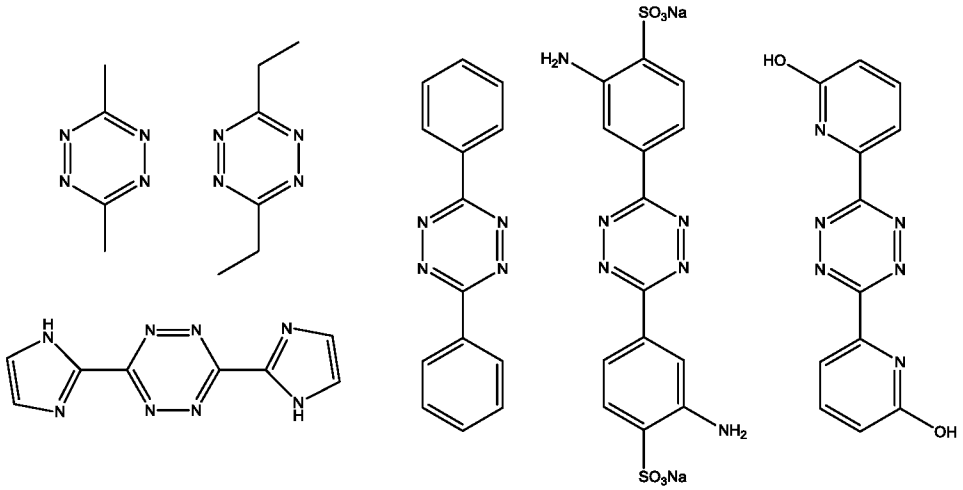
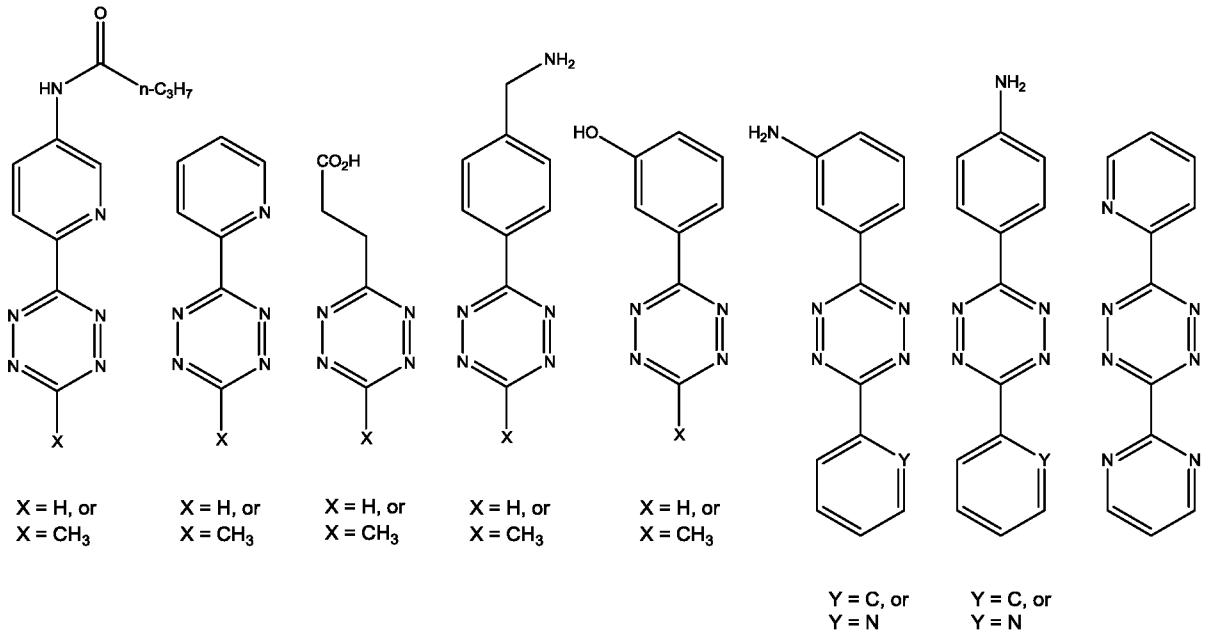
selected from OH, OR', SH, SR', NH_2, NHR', NR'_2, NH-CO-R', NR"-CO-R', NH-SO-R' or $NH-SO_2$-R' substituents, where R' represents a methyl, ethyl, phenyl or tolyl group and where R" represents a methyl or ethyl group. See below for some examples.



In case asymmetric 1,2,4,5-tetrazines are considered, one can choose any combination of given R^1 and R^2 residues that have been highlighted and listed above for the symmetric tetrazines according to formula (7), provided of course that R^1 and R^2 are different. Preferred asymmetric 1,2,4,5-tetrazines are those where at least one of the residues R^1 or R^2 is electron withdrawing in nature. Find below some example structures drawn.



Further considerations regarding the Activator

In the above the Activator has been described and defined with respect to either of two preferred embodiments of this invention, and for both embodiments 1,2-diazines, 1,2,4-triazines and 1,2,4,5-tetrazines, particularly 1,2,4,5-tetrazines, are the

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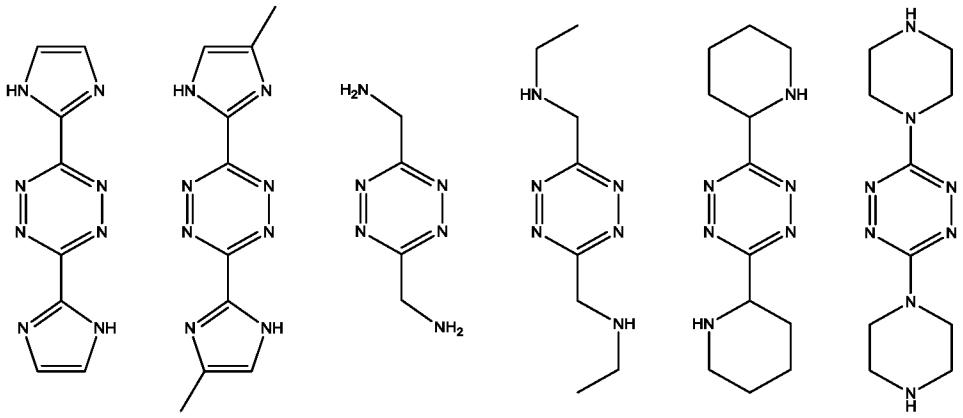
preferred diene Activators. In the below, some relevant features of the Activator will be highlighted, where it will also become apparent that there are plentiful options for designing the right Activator formulation for every specific application.

According to the invention, the Activator, e.g. a 1,2,4,5-tetrazine, has useful and beneficial pharmacological and pharmaco-kinetic properties, implying that the Activator is non-toxic or at least sufficiently low in toxicity, produces metabolites that are also sufficiently low in toxicity, is sufficiently soluble in physiological solutions, can be applied in aqueous or other formulations that are routinely used in pharmaceutics, and has the right log D value where this value reflects the hydrophilic/hydrophobic balance of the Activator molecule at physiological pH. As is known in the art, log D values can be negative (hydrophilic molecules) or positive (hydrophobic molecules), where the lower or the higher the log D values become, the more hydrophilic or the more hydrophobic the molecules are, respectively. Log D values can be predicted fairly adequately for most molecules, and log D values of Activators can be tuned by adding or removing polar or apolar groups in their designs. Find below some Activator designs with their corresponding calculated log D values (at pH = 7.4). Note that addition of methyl, cycloalkylene, pyridine, amine, alcohol or sulfonate groups or deletion of phenyl groups modifies the log D value, and that a very broad range of log D values is accessible.



log D =

- 0.50 - 0.10 - 3.07 - 1.33 - 0.09 - 3.42

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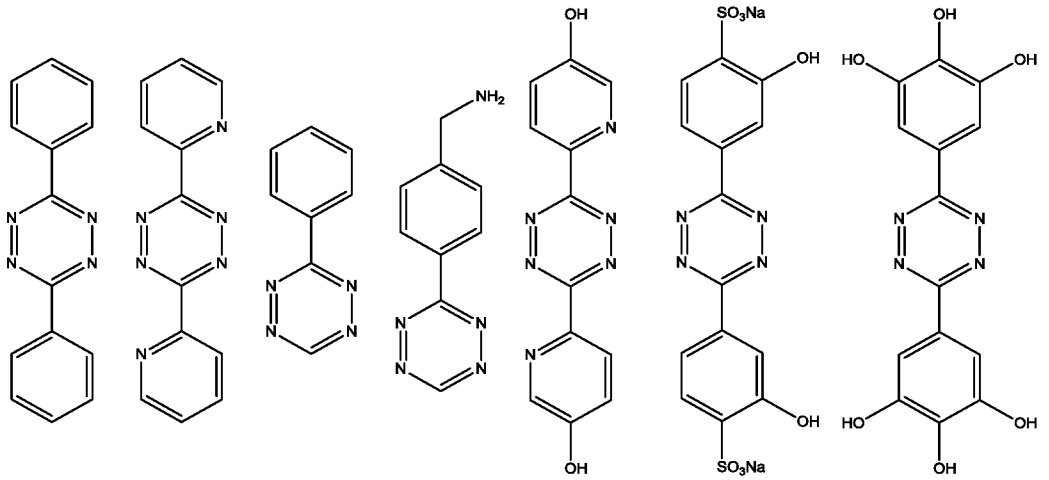


The given log D numbers have been calculated from a weighed method, with equal importance of the 'VG' (Viswanadhan, V. N.; Ghose, A. K.; Revankar, G. R.; Robins, R. K., J. Chem. Inf. Comput. Sci., 1989, 29, 163-172), 'KLOP' (according to Klopman, G.; Li, Ju-Yun.; Wang, S.; Dimayuga, M.: J.Chem.Inf.Comput.Sci., 1994, 34, 752) and 'PHYS' (according to the PHYSPROP© database) methods, based on an aqueous solution in 0.1 M in Na^+/K^+ Cl^-.

The Activator according to the invention has an appropriate reactivity towards the Prodrug, and this can be regulated by making the diene, particularly the 1,2,4,5-tetrazines, sufficiently electron deficient. Sufficient reactivity will ensure a fast retro Diels-Alder reaction with the Prodrug as soon as it has been reached by the Activator.

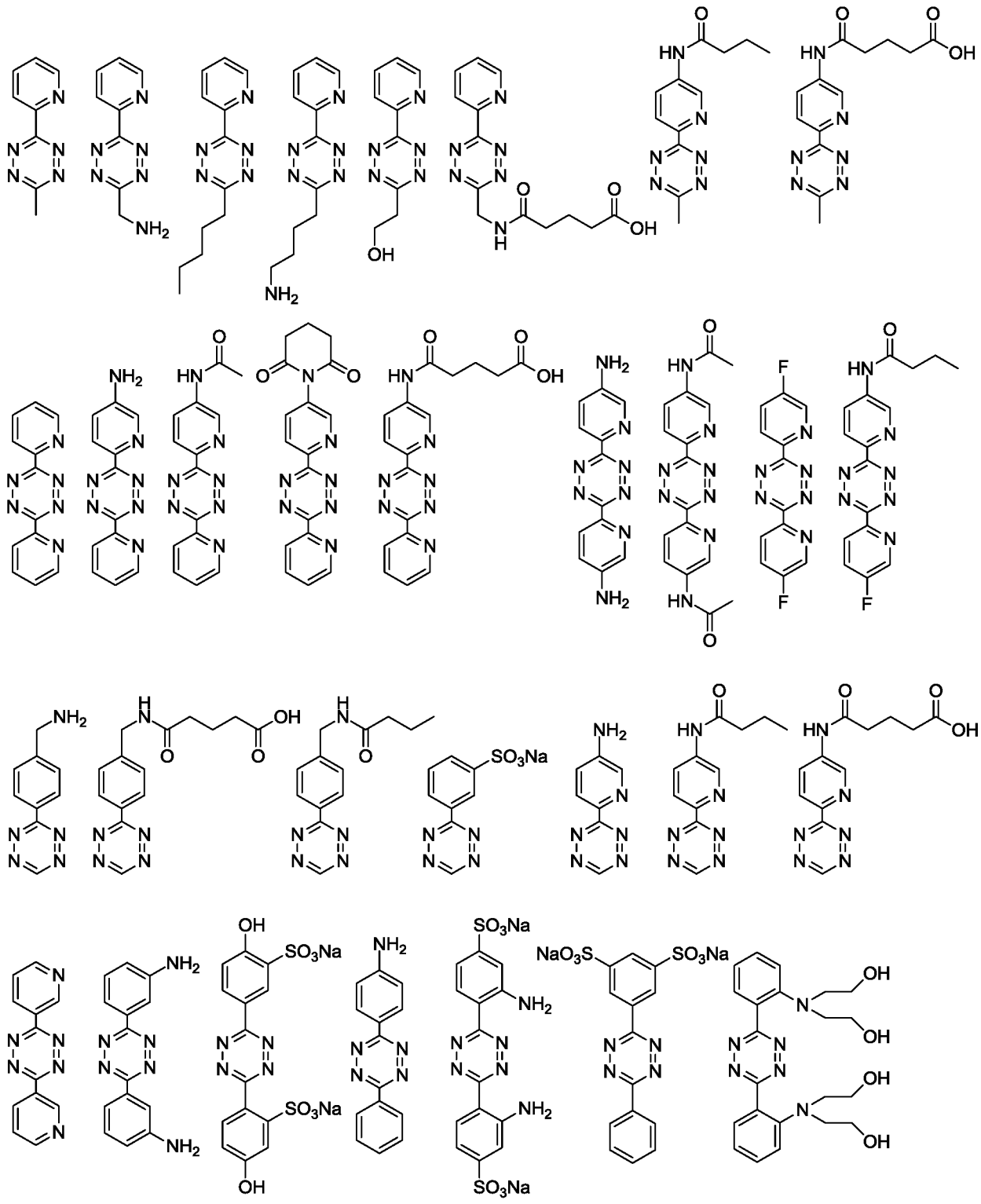
The Activator according to the invention has a good bio-availability, implying that it is available inside the (human) body for executing its intended purpose: effectively reaching the Prodrug at the Primary Target. Accordingly, the Activator does not stick significantly to blood components or to tissue that is non-targeted. The Activator may be designed to bind to albumin proteins that are present in the blood (so as to increase the blood circulation time, as is known in the art), but it should at the same time be released effectively from the blood stream to be able to reach the Prodrug. Accordingly, blood binding and blood releasing should then be balanced adequately. The blood circulation time of the Activator can also be increased by increasing the molecular weight of the Activator, e.g. by attaching polyethylene glycol (PEG) groups to the Activator ('pegylation'). Alternatively, the PK/PD of the activator may be modulated by conjugating the activator to another moiety such as a polymer, protein, (short) peptide, carbohydrate.

The Activator according to the invention may be multimeric, so that multiple diene moieties may be attached to a molecular scaffold, particularly to e.g. multifunctional

molecules, carbohydrates, polymers, dendrimers, proteins or peptides, where these scaffolds are preferably water soluble. Examples of scaffolds that can be used are (multifunctional) polyethylene glycols, poly (propylene imine) (PPI) dendrimers, PAMAM dendrimers, glycol based dendrimers, heparin derivatives, hyaluronic acid derivatives or serum albumine proteins such as HSA.

For applications where the prodrug activation is to occur in the extracellular domain, the diene is relatively hydrophilic.

Preferably, the Activator is a tetrazine selected from the following formulae:



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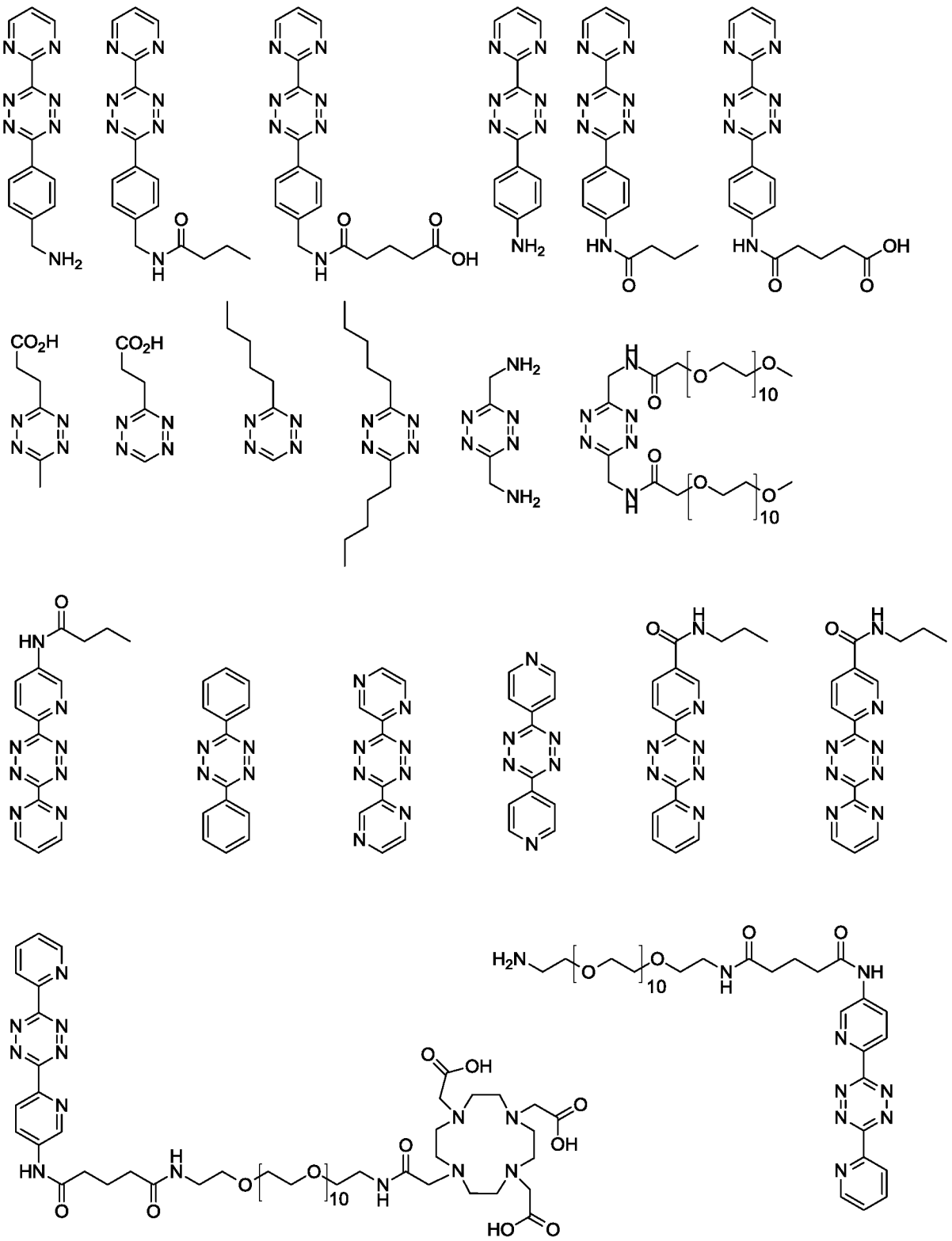

Depending on the position of the Prodrug (e.g. inside the cell or outside the cell; specific organ that is targeted) the Activator is designed to be able to effectively reach this Prodrug. Therefore, the Activator can for example be tailored by varying its log D value, its reactivity or its charge. The Activator may even be engineered with a targeting agent (e.g. a protein, a peptide and/or a sugar moiety), so that the Primary Target can be reached actively

instead of passively. In case a targeting agent is applied, it is preferred that it is a simple moiety (i.e. a short peptide or a simple sugar).

According to the invention, a mixture of different Activators can be applied. This may be relevant for regulation of the release profile of the drug.

The Activator that according to the invention will cause and regulate drug release at the Primary Target may additionally be modified with moieties giving extra function(s) to the Activator, either for in-vitro and/or for in-vivo studies or applications. For example, the Activator may be modified with dye moieties or fluorescent moieties (see e.g. S. Hilderbrand et al., Bioconjugate Chem., 2008, 19, 2297-2299 for 3-(4-benzylamino)-1,2,4,5-tetrazine that is amidated with the near-infrared (NIR) fluorophore VT680), or they may be functionalized with imaging probes, where these probes may be useful in imaging modalities, such as the nuclear imaging techniques PET or SPECT. In this way, the Activator will not only initiate drug release, but can also be localized inside the (human) body, and can thus be used to localize the Prodrug inside the (human) body. Consequently, the position and amount of drug release can be monitored. For example, the Activator can be modified with DOTA (or DTPA) ligands, where these ligands are ideally suited for complexation with $^{111}In^{3+}$-ions for nuclear imaging. In other examples, the Activator may be linked to ^{123}I or ^{18}F moieties, that are well established for use in SPECT or PET imaging, respectively. Furthermore, when used in combination with e.g. beta-emitting isotopes, such as Lu-177, or Y-90, prodrug activation can be combined with localized radiotherapy in a pretargeted format.

Synthesis routes to the above activators are readily available to the skilled person, based on standard knowledge in the art. References to tetrazine synthesis routes include Lions et al, *J. Org. Chem.*, 1965, 30, 318-319; Horwitz et al, *J. Am. Chem. Soc.*, 1958, 80, 3155-3159; Hapiot et al, *New. J. Chem.*, 2004, 28, 387-392, Kaim et al, *Z. Naturforsch.*, 1995, 50b, 123-127.

Prodrug

A Prodrug is a conjugate of the Drug D^D and the Trigger T^R and thus comprises a Drug that is capable of therapeutic action after its release from the Trigger. Such a Prodrug may optionally have specificity for disease targets.

The general formula of the Prodrug is shown below in Formula (8a) and (8b).

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$(Y^M)_k-(S^P)_t-(T^R)_m-(L^D)_n-(D^D)_r$ or $(T^R)_m-(L^D)_n(-(S^P)_t-(Y^M)_k)-(D^D)_r$

(8a) (8b)

The moiety Y^M can either be a targeting agent T^T or a masking moiety M^M; S^P is spacer; T^R is Trigger, L^D is linker, and D^D is drug.

For applications where drugs are released from a targeting agent: Y^M is a targeting agent T^T;

Formula (8a): $k = 1$; $m,r \geq 1$; $t,n \geq 0$.

Formula (8b): $k = 1$; $m,n,r \geq 1$; $t \geq 0$.

For applications where masked drugs are unmasked: Y^M is a masking moiety M^M;

Formula (8a) and (8b): $r = 1$; $m \geq 1$; $k,n,t \geq 0$.

Although it has been omitted for the sake of clarity in the above formula, D^D can further comprise T^T and/or M^M, optionally via S^P.

Drugs that can be used in a Prodrug relevant to this invention include but are not limited to: antibodies, antibody derivatives, antibody fragments, e.g. Fab2, Fab, scFV, diabodies, triabodies, antibody (fragment) fusions (eg bi-specific and trispecific mAb fragments), proteins, aptamers, oligopeptides, oligonucleotides, oligosaccharides, as well as peptides, peptoids, steroids, organic drug compounds, toxins, hormones, viruses, whole cells, phage. Typical drugs for which the invention is suitable include, but are not limited to: bi-specific and trispecific mAb fragments, immunotoxins, comprising eg ricin A, diphtheria toxin, cholera toxin. Other embodiments use auristatins, maytansines, calicheamicin, Duocarmycins, maytansinoids DM1 and DM4, auristatin MMAE, CC1065 and its analogs, camptothecin and its analogs, SN-38 and its analogs; antiproliferative/antitumor agents, antibiotics, cytokines, anti-inflammatory agents, anti-viral agents, antihypertensive agents, chemosensitizing and radiosensitizing agents. In other embodiments the released Drug D^D is itself a prodrug designed to release a further drug D^D. Drugs optionally include a membrane translocation moiety (adamantine, poly-lysine/argine, TAT) and/or a targeting agent (against eg a tumor cel receptor) optionally linked through a stable or labile linker.

Exemplary drugs for use as conjugates to the TCO derivative and to be released upon retro Diels Alder reaction with the Activator include but are not limited to: cytotoxic drugs, particularly those which are used for cancer therapy. Such drugs include, in general, DNA damaging agents, anti-metabolites, natural products and their analogs . Exemplary classes of cytotoxic agents include the enzyme inhibitors such as dihydrofolate reductase inhibitors, and thymidylate synthase inhibitors, DNA alkylators, radiation sensitizers, DNA intercalators, DNA cleavers, anti-tubulin agents, topoisomerases inhibitors, platinum-based drugs, the anthracycline family of drugs, the vinca drugs, the mitomycins, the bleomycins, the cytotoxic nucleosides, taxanes, lexitropsins, the pteridine family of drugs, diynenes, the podophyllotoxins, dolastatins, maytansinoids, differentiation inducers, and taxols. Particularly useful members of those classes include, for example, duocarmycin , methotrexate, methopterin, dichloromethotrexate, 5-fluorouracil DNA minor groove binders, 6-mercaptopurine, cytosine arabinoside, melphalan, leurosine, leurosideine, actinomycin, daunorubicin, doxorubicin, mitomycin C, mitomycin A, caminomycin, aminopterin, tallysomycin, podophyllotoxin and podophyllotoxin derivatives such as etoposide or etoposide phosphate, vinblastine, vincristine, vindesine, taxol, taxotere retinoic acid, butyric acid, N8-acetyl spermidine, camptothecin, calicheamicin, esperamicin, ene-diynes, and their analogues.

Exemplary drugs include the dolastatins and analogues thereof including: dolastatin A (U.S. Pat No. 4,486,414), dolastatin B (U.S. Pat No. 4,486,414), dolastatin 10 (U.S. Pat No. 4,486,444, 5,410,024, 5,504,191, 5,521,284, 5,530,097, 5,599,902, 5,635,483, 5,663,149, 5,665,860, 5,780,588, 6,034,065, 6,323,315),dolastatin 13 (U.S. Pat No. 4,986,988), dolastatin 14 (U.S. Pat No. 5,138,036), dolastatin 15 (U.S. Pat No. 4,879,278), dolastatin 16 (U.S. Pat No. 6,239,104), dolastatin 17 (U.S. Pat No. . 6,239,104), and dolastatin 18 (U.S. Pat No. . 6,239,104), each patent incorporated herein by reference in their entirety.

In exemplary embodiments of the invention, the drug moiety is a mytomycin, vinca alkaloid, taxol, anthracycline, a calicheamicin, maytansinoid or an auristatin.

It will be understood that chemical modifications may also be made to the desired compound in order to make reactions of that compound more convenient for purposes of preparing conjugates of the invention. Drugs containing an amine functional group for coupling to the TCO include mitomycin-C, mitomycin-A, daunorubicin, doxorubicin, aminopterin, actinomycin, bleomycin, 9-amino camptothecin, N8-acetyl spermidine, 1-(2

chloroethyl)1,2-dimethanesulfonyl hydrazide, tallysomycin, cytarabine, dolastatins (including auristatins) and derivatives thereof.

Drugs containing a hydroxyl function group for coupling to the TCO include etoposide, camptothecin, taxol, esperamicin, 1,8-dihydroxy-bicyclo[7.3.1]trideca-4-9-diene-2,6-diyne-13-one (U.S. Pat No. 5,198,560), podophyllotoxin, anguidine, vincristine, vinblastine, morpholine-doxorubicin, n-(5,5-diacetoxy-pentyl)doxorubicin, and derivatives thereof.

Drugs containing a sulfhydryl functional group for coupling to the TCO include esperamicin and 6-mecaptopurine, and derivatives thereof.

It will be understood that the drugs can optionally be attached to the TCO derivative through a linker L^D or a self-immolative linker L^D, or a combination thereof, and which may consist of multiple (self-immolative, or non immolative) units.

It will further be understood that one or more targeting agents T^T or masking moieties M^M may optionally be attached to the Drug D^D, Trigger T^R, or Linker L^D, optionally via a spacer or spacers S^P.

Several drugs may be replaced by an imageable label to measure drug targeting and release.

According to a further particular embodiment of the invention, the Prodrug is selected so as to target and or address a disease, such as cancer, an inflammation, an infection, a cardiovascular disease, e.g. thrombus, atherosclerotic lesion, hypoxic site, e.g. stroke, tumor, cardiovascular disorder, brain disorder, apoptosis, angiogenesis, an organ, and reporter gene/enzyme.

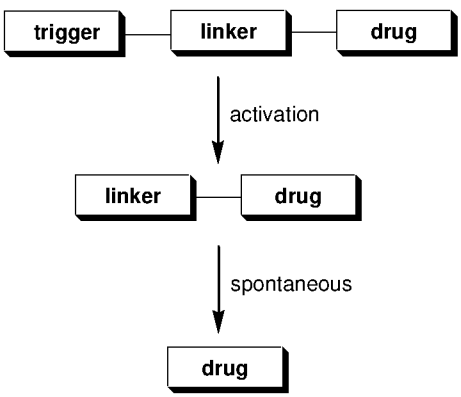
According to one embodiment, the Prodrug and/or the Activator can be multimeric compounds, comprising a plurality of Drugs and/or bioorthogonal reactive moieties. These multimeric compounds can be polymers, dendrimers, liposomes, polymer particles, or other polymeric constructs.

In the Prodrug, the Drug D^D and the Trigger T^R - the TCO derivative- can be directly linked to each other. They can also be bound to each other via a linker or a self-immolative linker L^D. It will be understood that the invention encompasses any conceivable manner in which the dienophile Trigger is attached to the Drug. The same holds for the attachment of an optional targeting agent T^T or masking moiety M^M to the Prodrug. Methods of affecting conjugation to these drugs, e.g. through reactive amino acids such as lysine or cysteine in the case of proteins, are known to the skilled person.

It will be understood that the drug moiety is linked to the TCO in such a way that the drug is eventually capable of being released after formation of the retro Diels-Alder adduct. Generally, this means that the bond between the drug and the TCO, or in the event of a linker, the bond between the TCO and the linker L^D, or in the event of a self-immolative linker L^D, the bond between the linker and the TCO and between the drug and the linker, should be cleavable. Predominantly, the drug and the optional linker is linked via a hetero-atom, preferably via O, N, NH, or S. The cleavable bond is preferably selected from the group consisting of carbamate, thiocarbamate, carbonate, ether, ester, amine, amide, thioether, thioester, sulfoxide, and sulfonamide bonds.

Thus, in the invention, linker concepts can be applied analogously to those known to the skilled person. Most reported prodrugs consist of three components: a trigger, a linker, and a parent drug, optionally a targeting molecule is attached to either the linker or the trigger. The trigger, which can e.g. be a substrate for a site-specific enzyme, or pH labile group, is often connected to the parent drug via a self-elimination linker. This linker is incorporated to facilitate enzymatic cleavage of the trigger, increasing active site accessibility and decreasing steric hindrance from the attached drug. Also the linker facilitates the straightforward use of a broad range of prodrugs in combination with the same trigger. Furthermore, the linker modulates prodrug stability, pharmacokinetics, organ distribution, enzyme recognition, and release kinetics. After trigger activation/removal, the linker must spontaneously eliminate to release the parent drug. Depending on the attached drug the linker or parts thereof can remain on the drug without impairing its action. The general concept is depicted in Scheme 2.

Scheme 2:



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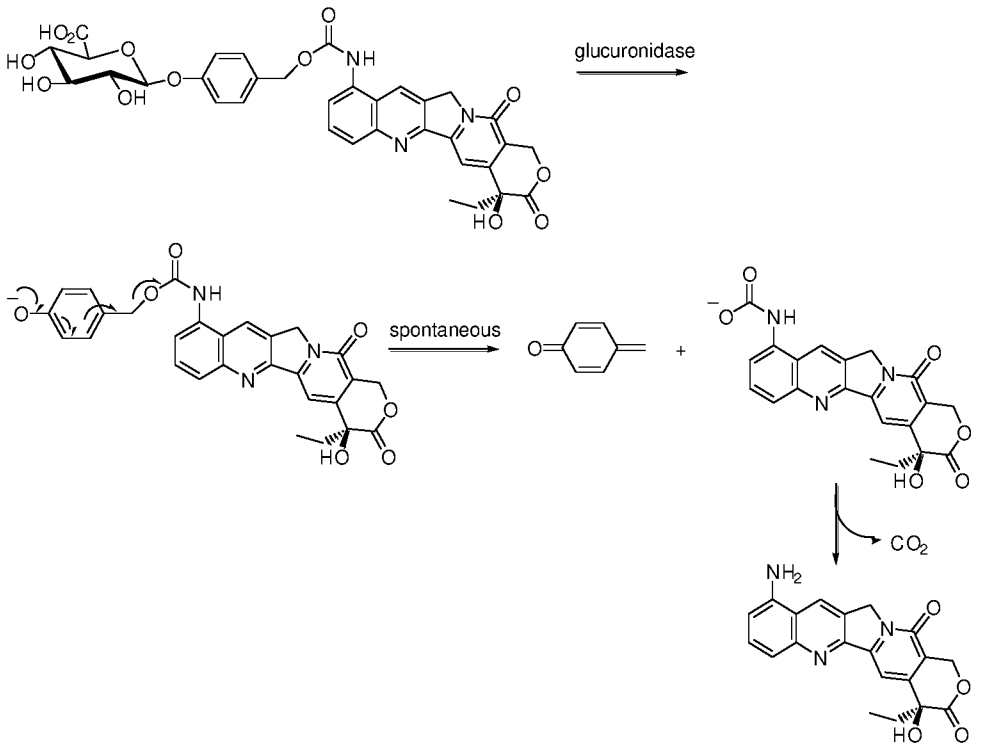
Two types of self-elimination linkers can be distinguished a) the electronic cascade linker b) the cyclization linker. The most prominent example of a cascade linker is the 1,6 elimination spacer shown in Scheme 3 in a β-glucuronide prodrug of anticancer agent 9-aminocamptothecin. After unmasking of the aromatic hydroxyl function by the enzyme β-glucuronidase (present in certain necrotic tumor areas), this group becomes electron-donating and initiates an electronic cascade that leads to expulsion of the leaving group, which releases the free drug after elimination of CO_2. This cascade, based on a quinone-methide rearrangement, can also be initiated by the lone pair of an unmasked amine or thiol instead of the hydroxyl. The formed quinone-methide species is trapped by water to form a phenol derivative.

Scheme 3:



Some other trigger-linker concepts are depicted in Scheme 4. The trigger in A is activated by plasmatic esterases. Hydrolysis of the tert-butyl ester affords the free aromatic hydroxyl group, which starts the quinone-methide cascade. This construct has been targeted by conjugation to an antibody (R). In B, the hydrolysis of cephalosporins by beta-lactamase enzymes is used as a trigger. Hydrolysis of the lactam ring can to lead expulsion of the drug substituent depending on its leaving group nature. Drugs have been conjugated via an ester,

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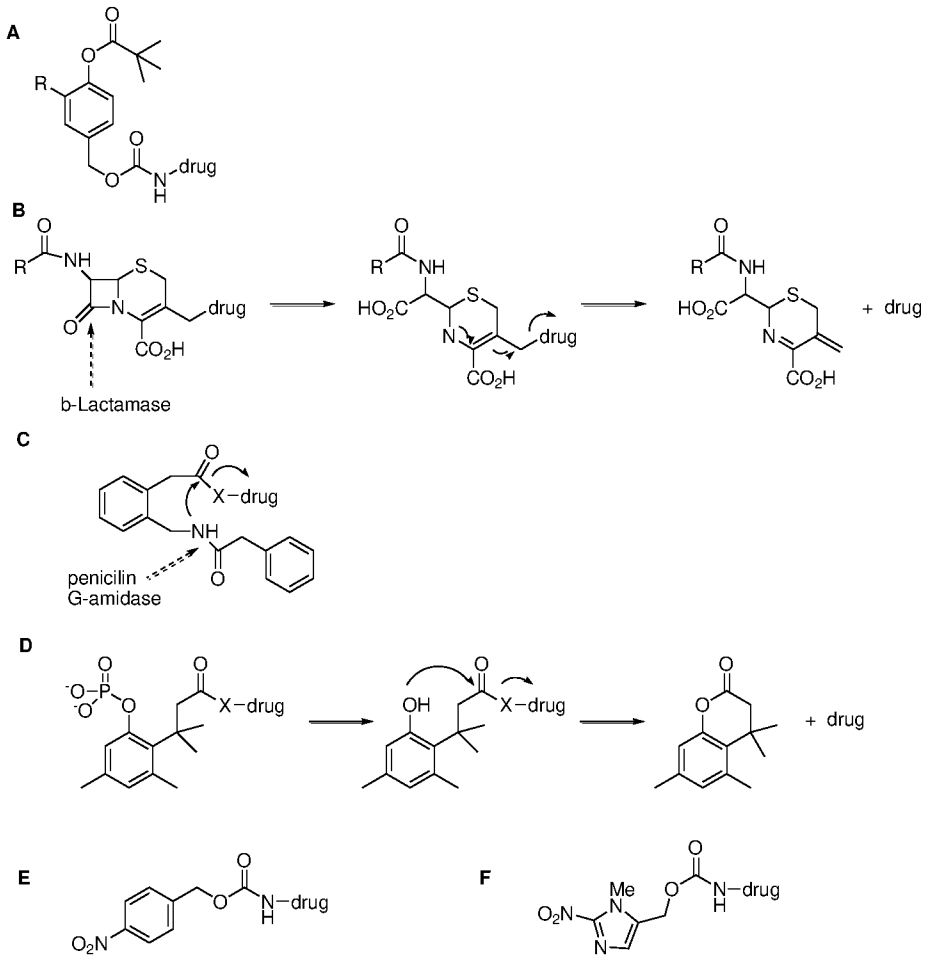
amide, sulfide, amine and carbamate link. Two examples of aromatic cyclization-based linkers are C and D. In C cleavage by penicillin G-amidase leads to intramolecular attack of the amine on the carbonyl, releasing the drug. D shows a phosphatase-sensitive prodrug. Cleavage of the phosphate by human alkaline phosphatase affords a hydroxyl that reacts to a lactam by releasing the drug. In E an example is shown of a prodrug that it triggered by the reduction of a nitro group to an amine. This reduction can be performed by nitroreductase in the presence of NADPH. Furthermore, a number of heterocyclic nitro constructs are known (F) that are reduced in hypoxic (tumor) tissue and, hence, can initiate a cascade without the assistance of an enzyme. Other triggers used in prodrug therapy are sensitive to plasmin, tyrosine hydroxylase (highly expressed in neuroblastoma), tyrosinase or cathepsin B.

Scheme 4: X = O, N, S



<u>The combination of and reaction between the TCO-Trigger and the Activator</u>

The following schemes depict non-limiting examples illustrative for the various mechanisms that can be made to apply on the basis of the choice for the rDA reaction for activating a prodrug. Note that in cases of release of amine functional drugs these can be e.g. primary or secondary amine, aniline, imidazole or pyrrole type of drugs, so that the drug may be varying in leaving group character. Release of drugs with other functionalities may also be possible (e.g. thiol functinalized drugs), in case corresponding hydrolytically stable TCO prodrugs are applied. The drawn fused ring products may or may not tautomerize to other more favorable tautomers.

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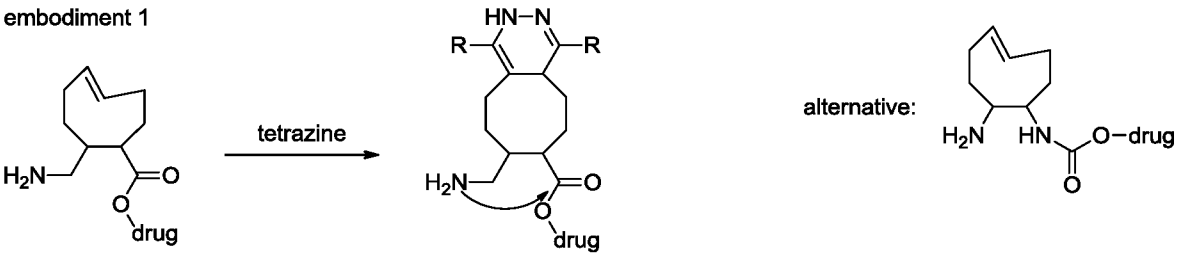
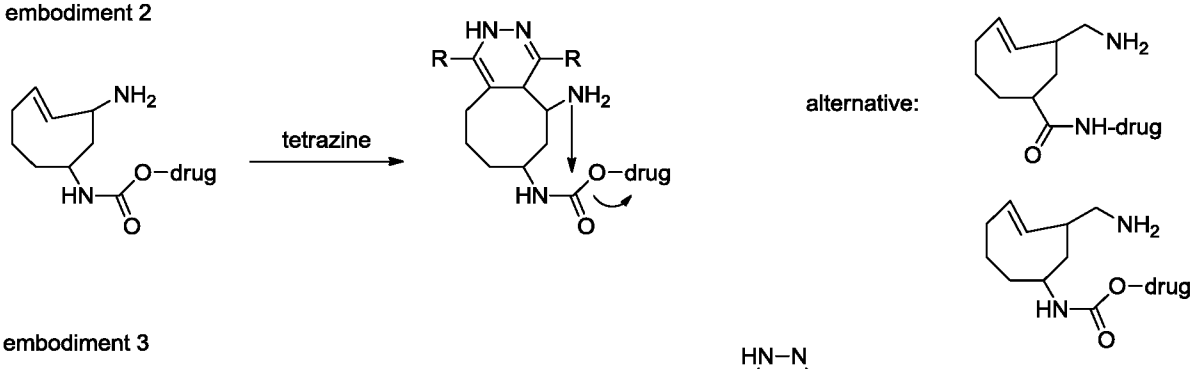
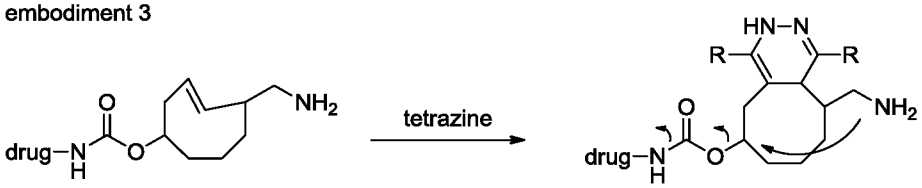
embodiment 1



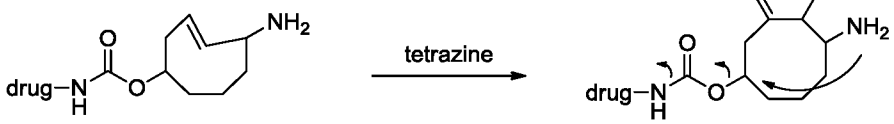
embodiment 2



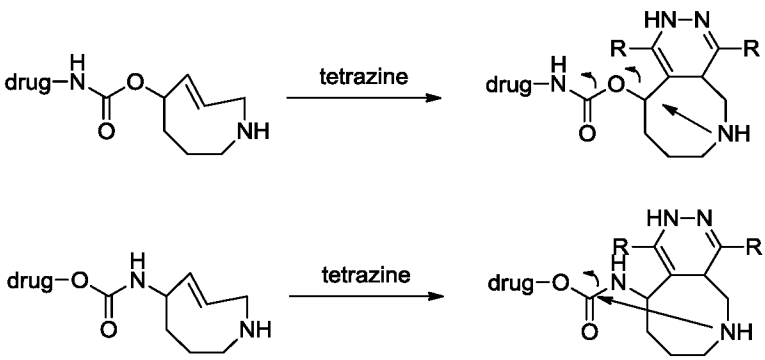
embodiment 3



embodiment 4



embodiment 5



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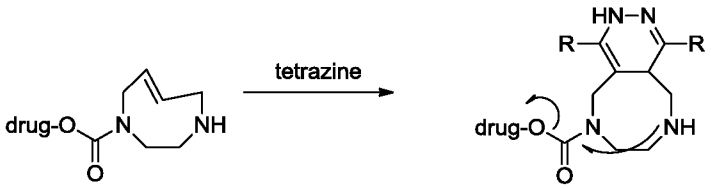
embodiment 6



In a preferred embodiment, the drug is provided in the form of an antibody-toxin conjugate. The conjugate is provided with a TCO moiety as identified above, so as to enable bio-orthogonal chemically activated toxin release. In another embodiment, the drug is a bi- or trispecific antibody derivative that serves to bind to tumor cells and recruit and activate T-cells, the T-cell binding function of which is inactivated by being linked to a TCO moiety as described above. The latter, again, serving to enable bio-orthogonal chemically activated drug activation.

Targeting

The kits and method of the invention are very suitable for use in targeted delivery of drugs.

A "primary target" as used in the present invention relates to a target for a targeting agent for therapy. For example, a primary target can be any molecule, which is present in an organism, tissue or cell. Targets include cell surface targets, e.g. receptors, glycoproteins; structural proteins, e.g. amyloid plaques; abundant extracullular targets such as stroma; extracellular matrix targets such as growth factors, and proteases; intracellular targets, e.g. surfaces of Golgi bodies, surfaces of mitochondria, RNA, DNA, enzymes, components of cell signaling pathways; and/or foreign bodies, e.g. pathogens such as viruses, bacteria, fungi, yeast or parts thereof. Examples of primary targets include compounds such as proteins of which the presence or expression level is correlated with a certain tissue or cell type or of which the expression level is up regulated or down-regulated in a certain disorder. According to a particular embodiment of the present invention, the primary target is a protein such as a (internalizing or non-internalizing) receptor.

According to the present invention, the primary target can be selected from any suitable targets within the human or animal body or on a pathogen or parasite, e.g. a group comprising cells such as cell membranes and cell walls, receptors such as cell membrane receptors, intracellular structures such as Golgi bodies or mitochondria, enzymes, receptors, DNA, RNA, viruses or viral particles, antibodies, proteins, carbohydrates,

monosacharides, polysaccharides, cytokines, hormones, steroids, somatostatin receptor, monoamine oxidase, muscarinic receptors, myocardial sympatic nerve system, leukotriene receptors, e.g. on leukocytes, urokinase plasminogen activator receptor (uPAR), folate receptor, apoptosis marker, (anti-)angiogenesis marker, gastrin receptor, dopaminergic system, serotonergic system, GABAergic system, adrenergic system, cholinergic system, opoid receptors, GPIIb/IIIa receptor and other thrombus related receptors, fibrin, calcitonin receptor, tuftsin receptor, integrin receptor, fibronectin, VEGF/EGF and VEGF/EGF receptors, TAG72, CEA, CD19, CD20,CD22, CD40, CD45, CD74, CD79, CD105, CD138, CD174, CD227, CD326, CD340, MUC1, MUC16, GPNMB, PSMA, Cripto, Tenascin C, Melanocortin-1 receptor , CD44v6, G250, HLA DR, ED-B, TMEFF2 , EphB2, EphA2, FAP, Mesothelin, GD2, CAIX, 5T4, matrix metalloproteinase (MMP), P/E/L-selectin receptor, LDL receptor, P-glycoprotein, neurotensin receptors, neuropeptide receptors, substance P receptors, NK receptor, CCK receptors, sigma receptors, interleukin receptors, herpes simplex virus tyrosine kinase, human tyrosine kinase. In order to allow specific targeting of the above-listed primary targets, the targeting agent T^T can comprise compounds including but not limited to antibodies, antibody fragments, e.g. Fab2, Fab, scFV, diabodies, triabodies, VHH, antibody (fragment) fusions (eg bi-specific and trispecific mAb fragments), proteins, peptides, e.g. octreotide and derivatives, VIP, MSH, LHRH, chemotactic peptides, bombesin, elastin, peptide mimetics, carbohydrates, monosacharides, polysaccharides, viruses, whole cells, drugs, polymers, liposomes, chemotherapeutic agents, receptor agonists and antagonists, cytokines, hormones, steroids. Examples of organic compounds envisaged within the context of the present invention are, or are derived from, estrogens, e.g. estradiol, androgens, progestins, corticosteroids, methotrexate, folic acid, and cholesterol. In a preferred embodiment, the targeting agent T^T is an antibody. According to a particular embodiment of the present invention, the primary target is a receptor and a targeting agent is employed, which is capable of specific binding to the primary target. Suitable targeting agents include but are not limited to, the ligand of such a receptor or a part thereof which still binds to the receptor, e.g. a receptor binding peptide in the case of receptor binding protein ligands. Other examples of targeting agents of protein nature include interferons, e.g. alpha, beta, and gamma interferon, interleukins, and protein growth factor, such as tumor growth factor, e.g. alpha, beta tumor growth factor, platelet-derived growth factor (PDGF), uPAR targeting protein, apolipoprotein, LDL, annexin V, endostatin, and angio statin. Alternative examples of targeting agents include DNA, RNA, PNA and LNA which are e.g. complementary to the primary target.

According to a further particular embodiment of the invention, the primary target and targeting agent are selected so as to result in the specific or increased targeting of a tissue or disease, such as cancer, an inflammation, an infection, a cardiovascular disease, e.g. thrombus, atherosclerotic lesion, hypoxic site, e.g. stroke, tumor, cardiovascular disorder, brain disorder, apoptosis, angiogenesis, an organ, and reporter gene/enzyme. This can be achieved by selecting primary targets with tissue-, cell- or disease- specific expression. For example, membrane folic acid receptors mediate intracellular accumulation of folate and its analogs, such as methotrexate. Expression is limited in normal tissues, but receptors are overexpressed in various tumor cell types.

Masking Moieties

Masking moieties M^M can be a protein, peptide, polymer, polyethylene glycol, carbohydrate, organic construct, that further shield the bound drug D^D or Prodrug. This shielding can be based on eg steric hindrance, but it can also be based on a non covalent interaction with the drug D^D. Such masking moiety may also be used to affect the in vivo properties (eg blood clearance; recognition by the immunesystem) of the drug D^D or Prodrug.

Spacers

Spacers S^P include but are not limited to polyethylene glycol (PEG) chains varying from 2 to 200, particularly 3 to 113 and preferably 5-50 repeating units. Other examples are biopolymer fragments, such as oligo- or polypeptides or polylactides. Further preferred examples are shown in Example 9.

Administration

In the context of the invention, the Prodrug is usually administered first, and it will take a certain time period before the Prodrug has reached the Primary Target. This time period may differ from one application to the other and may be minutes, days or weeks. After the time period of choice has elapsed, the Activator is administered, will find and react with the Prodrug and will thus activate Drug release at the Primary Target.

The compositions of the invention can be administered via different routes including intravenous injection, intraperatonial, oral administration, rectal administration and inhalation. Formulations suitable for these different types of administrations are known to the skilled person. Prodrugs or Activators according to the invention can be administered together with a pharmaceutically acceptable carrier. A suitable pharmaceutical carrier as used

herein relates to a carrier suitable for medical or veterinary purposes, not being toxic or otherwise unacceptable. Such carriers are well known in the art and include saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. The formulation should suit the mode of administration.

It will be understood that the chemical entities administered, viz. the prodrug and the activator, can be in a modified form that does not alter the chemical functionality of said chemical entity, such as salts, hydrates, or solvates thereof.

After administration of the Prodrug, and before the administration of the Activator, it is preferred to remove excess Prodrug by means of a Clearing Agent in cases when prodrug activation in circulation is undesired and when natural prodrug clearance is insufficient. A Clearing Agent is an agent, compound, or moiety that is administered to a subject for the purpose of binding to, or complexing with, an administered agent (in this case the Prodrug) of which excess is to be removed from circulation. The Clearing Agent is capable of being directed to removal from circulation. The latter is generally achieved through liver receptor-based mechanisms, although other ways of secretion from circulation exist, as are known to the skilled person. In the invention, the Clearing Agent for removing circulating Prodrug, preferably comprises a diene moiety, e.g. as discussed above, capable of reacting to the TCO moiety of the Prodrug.

EXAMPLES

The following examples demonstrate the invention or aspects of the invention, and do not serve to define or limit the scope of the invention or its claims.

Methods. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Varian Mercury (400 MHz for ^{1}H-NMR and 100 MHz for ^{13}C-NMR) spectrometer at 298 K. Chemical shifts are reported in ppm downfield from TMS at room temperature. Abbreviations used for splitting patterns are s = singlet, t = triplet, q = quartet, m = multiplet and br = broad. IR spectra were recorded on a Perkin Elmer 1600 FT-IR (UATR). LC-MS was performed using a Shimadzu LC-10 AD VP series HPLC coupled to a diode array detector (Finnigan Surveyor PDA Plus detector, Thermo Electron Corporation) and an Ion-Trap (LCQ Fleet, Thermo Scientific). Analyses were performed using a Alltech Alltima HP C_{18} 3μ column using an injection volume of 1-4 μL, a flow rate of 0.2 mL min^{-1} and typically a gradient (5% to 100% in 10 min, held at 100% for a further 3 min) of CH_3CN in H_2O (both containing 0.1% formic acid) at 25 °C. Preparative RP-HPLC (CH_3CN / H_2O with 0.1% formic acid) was performed

using a Shimadzu SCL-10A VP coupled to two Shimadzu LC-8A pumps and a Shimadzu SPD-10AV VP UV-vis detector on a Phenomenex Gemini 5µ C_{18} 110A column. Size exclusion (SEC) HPLC was carried out on an Agilent 1200 system equipped with a Gabi radioactive detector. The samples were loaded on a Superdex-200 10/300 GL column (GE Healthcare Life Sciences) and eluted with 10 mM phosphate buffer, pH 7.4, at 0.35-0.5 mL/min. The UV wavelength was preset at 260 and 280 nm. The concentration of antibody solutions was determined with a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific) from the absorbance at 322 nm and 280 nm, respectively.

Materials. All reagents, chemicals, materials and solvents were obtained from commercial sources, and were used as received: Biosolve, Merck and Cambridge Isotope Laboratories for (deuterated) solvents; and Aldrich, Acros, ABCR, Merck and Fluka for chemicals, materials and reagents. All solvents were of AR quality. 4-(*t*-Butyldimethylsilyloxymethyl)-2,6-dimethylphenol was synthesized according to a literature procedure (Y. H. Choe, C. D. Conover, D. Wu, M. Royzen, Y. Gervacio, V. Borowski, M. Mehlig, R. B. Greenwald, *J. Controlled Release* **2002**, *79*, 55–70). Doxorubicin hydrochloride was obtained from Avachem Scientific.

Example 1

Synthesis of tetrazine Activators

General procedures

Apart from the tetrazines described in detail below, a series of other tetrazines has been prepared. Pinner-type reactions have been used, where the appropriate nitriles have been reacted with hydrazine to make the dihydro 1,2,4,5-tetrazine intermediates. Instead of nitriles, amidines have also been used as reactants, as it is known in the art. The use of sulfur in this reaction is also known, as in some cases this aids the formation of the dihydro 1,2,4,5-tetrazine. Oxidation of this intermediate results in the tetrazine diene Activators. The below reactions describe some of the prepared tetrazines and illustrate some of the possibilities (e.g. use of solvent, concentrations, temperature, equivalents of reactants, options for oxidation, etc.) to make and isolate tetrazines. Other methods known in the art may also be used to prepare tetrazines or other Activators.

*Synthesis of 3,6-bis(2-pyridyl)-1,2,4,5-tetrazine (**2**)*

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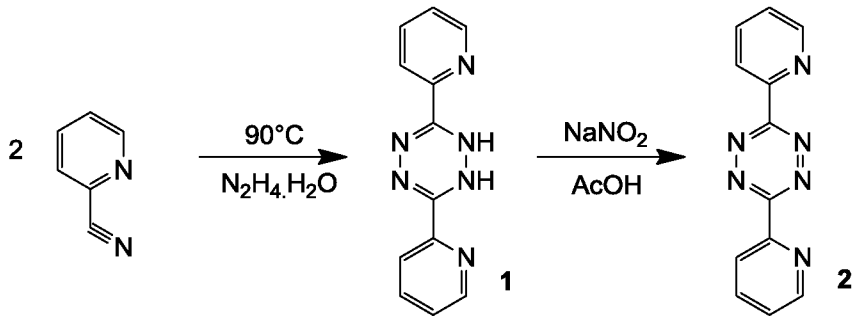
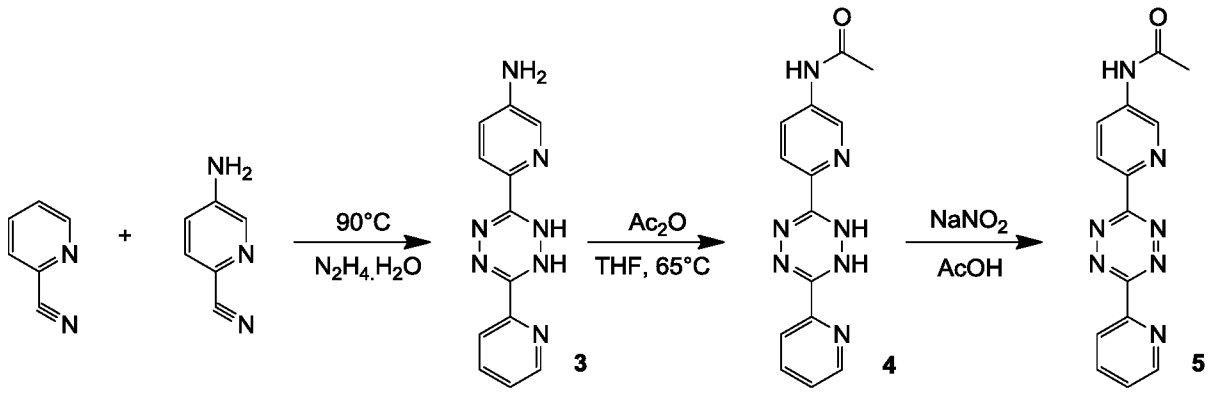


2-Cyanopyridine (10.00 g, 96.0 mmol) and hydrazine hydrate (15.1 g; 300 mmol) were stirred overnight at 90 °C in an inert atmosphere. The turbid mixture was cooled to room temperature, filtered, and the residue was subsequently washed with water (20 mL) and ethanol (20 mL), and dried in vacuo to yield the crude dihydrotetrazine **1** as an orange solid (7.35 g; 65%).

The dihydrotetrazine (**1**, 100 mg; 0.419 mmol) was suspended in acetic acid (3 mL), and sodium nitrite (87 mg; 1.26 mmol) was added. An immediate color change from orange to dark red was observed, and the oxidized product was isolated by filtration. The residue was washed with water (10 mL) and dried in vacuo to yield the title compound as a purple solid (**2**, 92 mg; 93%).

^{1}H NMR ($CDCl_3$): δ = 9.00 (d, 2H), 8.76 (d, 2H), 8.02 (t, 2H), 7.60 (dd, 2H) ppm. ^{13}C NMR ($CDCl_3$): δ = 163.9, 151.1, 150.1, 137.5, 126.6, 124.5 ppm. HPLC-MS/PDA: one peak in chromatogram, m/z = 237.00 ($M+H^+$), λ_{max} = 296 and 528 nm.

*Synthesis of 3-(5-acetamido-2-pyridyl)-6-(2-pyridyl)-1,2,4,5-tetrazine (**5**)*



2-Cyanopyridine (5.00 g, 48.0 mmol), 5-amino-2-cyanopyridine (5.72 g; 48.0 mmol) and hydrazine hydrate (15.1 g; 300 mmol) were stirred overnight at 90 °C in an inert atmosphere. The turbid mixture was cooled to room temperature, filtered, and the residue was

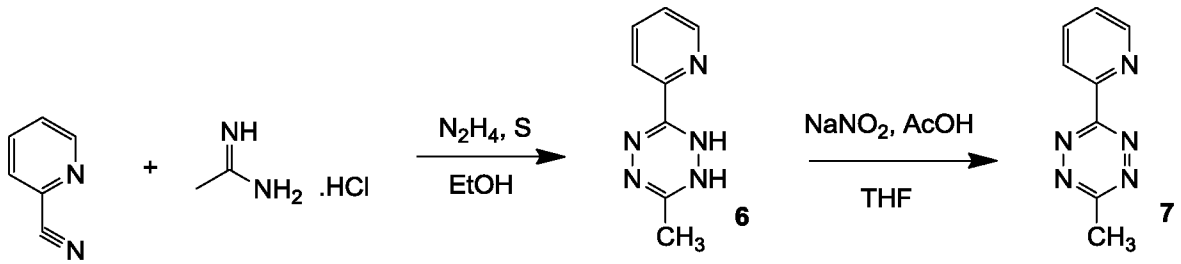
subsequently washed with water (20 mL) and ethanol (20 mL), and dried in vacuo. The orange solid was suspended in acetone (200 mL), impregnated onto silica gel (20 g), and chromatographed using a gradient (0% to 70%) of acetone and heptane, to yield dihydrotetrazine **3** as an orange solid (1.46 g; 12% yield).

The dihydrotetrazine (**3**, 90 mg; 0.355 mmol) was dissolved in THF (1 mL), and acetic anhydride (54.4 mg; 0.533 mmol) was added. The solution was heated to reflux in an inert atmosphere for 18 hr. The orange precipitate was isolated by filtration, and washed with THF (3 mL) to give the acetamide of the dihydrotetrazine (**4**, 90 mg; 86% yield).

Acetamide **4** (50 mg, 0.169 mmol) was suspended in acetic acid (1 mL), and sodium nitrite (35 mg; 0.508 mmol) was added. An immediate color change from orange to dark red was observed, and the oxidized product was isolated by filtration. The residue was washed with water (5 mL) and dried in vacuo to yield the title compound as a purple solid (**5**, 42 mg; 84%).

^{1}H NMR (DMSO-d_6): δ = 9.03 (d, 1H), 8.93 (d, 1H), 8.61 (dd, 2H), 8.42 (dd, 1H), 8.16 (dt, 1H), 7.73 (dd, 1H), 2.17 (s, 3H) ppm. ^{13}C NMR (DMSO-d_6): δ = 169.5, 163.0, 162.8, 150.6, 150.2, 143.8, 141.2, 138.5, 137.8, 126.6, 126.1, 124.9, 124.2, 24.1 ppm. HPLC-MS/PDA: one peak in chromatogram, m/z = 293.9 ($M+H^+$), λ_{max} = 323 and 529 nm.

Synthesis of 3-(2-pyridyl)-6-methyl-1,2,4,5-tetrazine (7)



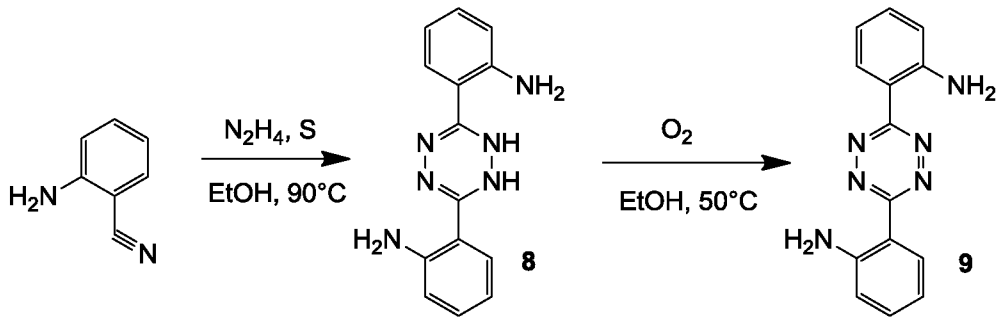
2-Cyanopyridine (500 mg, 4.8 mmol), acetamidine hydrochloride (2.00 g, 21.2 mmol) and sulfur (155 mg, 4.8 mmol) were stirred in ethanol (5 mL) under an inert atmosphere of argon. Hydrazine hydrate (2.76 g; 55.2 mmol) was added and the mixture was stirred overnight at 20 °C. The turbid mixture was filtered and the filtrate was evaporated to dryness, to yield 2.9 g of orange colored crude product **6**.

Subsequently, **6** (800 mg) was suspended in a mixture of THF (3 mL) and acetic acid (4 mL). A solution of $NaNO_2$ (2.0 g; 29.0 mmol) in water (3 mL) was added at 0°C. Instantaneous coloration to a red/purple suspension was observed. After 5 min of stirring at 0°C, chloroform and water were added. The purple chloroform layer was washed

twice with water and then concentrated. The solid residue was stirred in a 1:1 mixture of chloroform and hexane, and then filtered. The filtrate was concentrated and the crude product was purified by silica column chromatography applying chloroform/acetone mixtures as eluent, yielding pure product **7** (48 mg, 21% yield overall, as calculated from 2-cyanopyridine).

^{1}H NMR ($CDCl_3$): δ = 8.96 (d, 1H), 8.65 (d, 1H), 7.99 (t, 1H), 7.56 (dd, 1H), 3.17 (s, 3H) ppm. ^{13}C NMR ($CDCl_3$): δ = 168.1, 163.6, 150.9, 150.3, 137.4, 126.3, 123.9, 21.4 ppm. HPLC-MS/PDA: one peak in chromatogram, m/z = 174.3 ($M+H^+$), λ_{max} = 274 and 524 nm.

*Synthesis of 3,6-bis(2-aminophenyl)-1,2,4,5-tetrazine (**9**)*

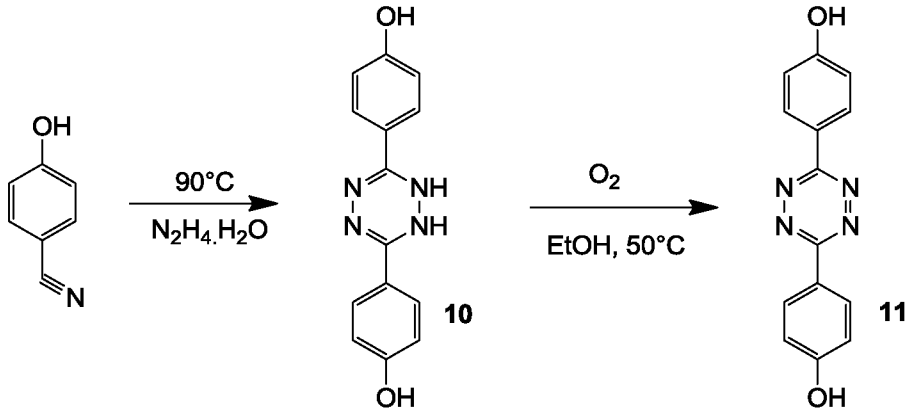


2-Aminobenzonitrile (1.00 g; 8.46 mmol) was dissolved in ethanol (3 mL) and hydrazine hydrate (2.06 g; 41.2 mmol) was added. The mixture was cooled to 0°C and sulfur (0.17 g; 5.30 mmol) was added. Stirring was continued for 15 min, and subsequently the mixture was heated at 90°C. After 3 hr, the yellow precipitate was isolated by filtration, washed with ethanol (10 mL), and subsequently triturated twice with chloroform (2 times 10 mL), to yield the yellow intermediate **8** (343 mg, 30%).

Intermediate **8** (105 mg; 0.394 mmol) was dissolved in ethanol (15 mL), and oxygen was bubbled through this solution at 50°C. Within minutes, the color changed from yellow to dark orange/red, and a precipitate was formed. After 2 hr, the precipitate was filtered, washed with ethanol and dried to give the product **9** as dark red crystals (89 mg, 86%).

^{1}H NMR (DMSO-d_6): δ = 8.39 (d, 2H), 7.32 (t, 2H), 7.04 (s, 4H), 6.93 (d, 2H), 6.75 (t, 2H) ppm. ^{13}C NMR (DMSO-d_6): δ = 162.7, 149.6, 133.0, 129.0, 117.1, 115.8, 111.6 ppm. HPLC-MS/PDA: one peak in chromatogram, m/z = 265.4 ($M+H^+$), λ_{max} = 237, 293, 403 and 535 nm.

*Synthesis of 3,6-bis(4-hydroxyphenyl)-1,2,4,5-tetrazine (**11**)*

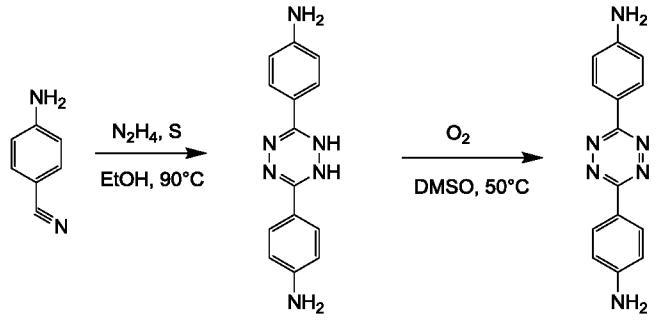


4-Hydroxybenzonitrile (1.06 g; 8.90 mmol) was dissolved in hydrazine hydrate (3.09 g; 61.7 mmol), and the mixture was heated to 90°C for 16 hr. The yellow precipitate was filtered and washed with water (25 mL) and ethanol (10 mL), to yield crude intermediate **10** as a yellow powder (870 mg; 62%).

The intermediate (**10**, 173 mg; 0.645 mmol) was suspended in ethanol (10 mL), and oxygen was bubbled through this mixture at 50°C. Within minutes, the color changed from yellow to dark orange/red. After 6 hr, the precipitate was filtered, washed with ethanol and dried, to give the product **11** as dark red crystals (136 mg, 80%).

^{1}H NMR (DMSO-d_6): δ = 10.35 (br. s, 2H), 8.36 (d, 4H), 7.02 (d, 4H) ppm. ^{13}C NMR (DMSO-d_6): δ = 162.6, 161.5, 129.2, 122.6, 116.3 ppm. HPLC-MS/PDA: one peak in chromatogram, m/z = 267.1 ($M+H^+$), λ_{max} = 235, 330 and 535 nm.

*Synthesis of 3,6-bis(4-aminophenyl)-1,2,4,5-tetrazine (**13**)*



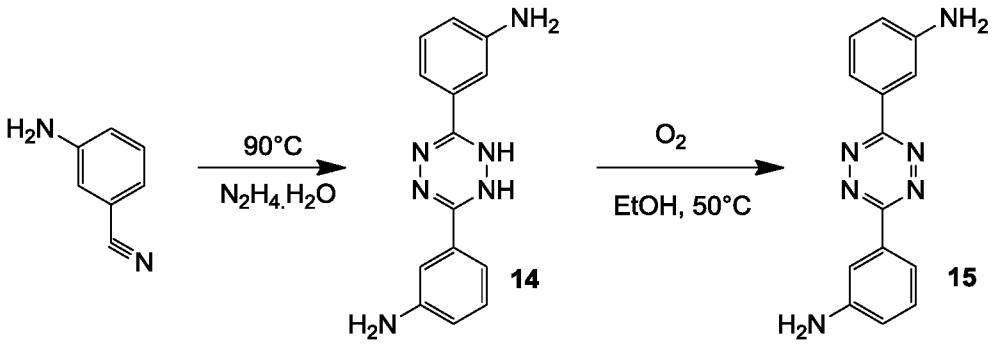
4-Aminobenzonitrile (1.00 g; 8.46 mmol) was dissolved in ethanol (3 mL), and subsequently hydrazine hydrate (2.12 g; 42.2 mmol) and sulfur (0.176 g; 5.5 mmol) were added. The mixture was heated at 90°C for 90 min, and the yellow precipitate was isolated by

filtration, washed with ethanol (10 mL), and subsequently triturated with acetone (12 mL) to yield the yellow intermediate **12** (190 mg, 17%).

Intermediate **12** (50 mg; 0.188 mmol) was dissolved in DMSO (1 mL), and oxygen was bubbled through this solution at 20°C. After 5 hr, the reaction mixture was poured in brine (13 mL), and the red precipitate was filtered off, washed with water (10 mL), and dried *in vacuo*. The red powder was further purified by trituration with acetone (15 mL), to yield product **13** as a red solid (13.7 mg, 27%).

^{1}H NMR (DMSO-d_6): δ = 8.17 (d, 2H), 7.75 (d, 2H), 6.02 (s, 4H) ppm. ^{13}C NMR (DMSO-d_6): δ = 162.3, 152.8, 128.5, 118.3, 113.8 ppm. HPLC-MS/PDA: one peak in chromatogram, m/z = 265.2 ($M+H^+$), λ_{max} = 241, 370 and 530 nm.

*Synthesis of 3,6-bis(3-aminophenyl)-1,2,4,5-tetrazine (**15**)*

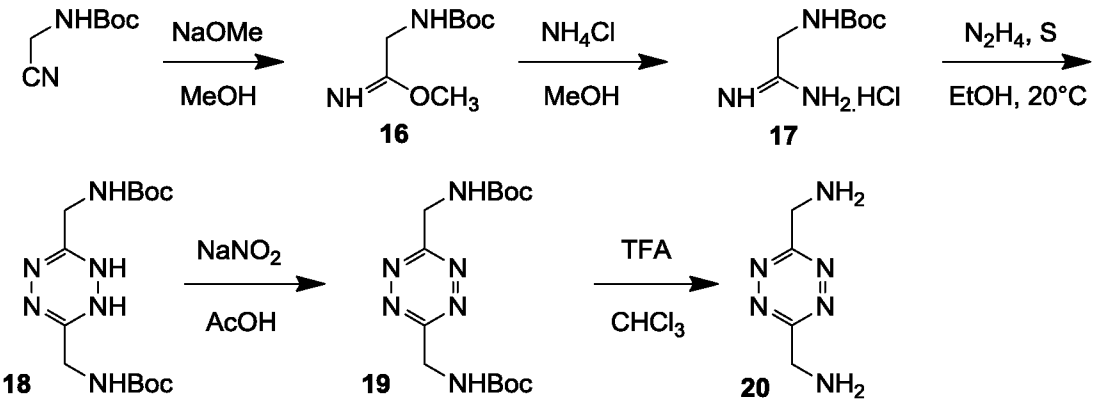


3-Aminobenzonitrile (1.00 g; 8.460 mmol) was dissolved in hydrazine hydrate (2.50 mL; 51.4 mmol), and the mixture was heated to 90°C for 3 days. Water (5 mL) was added, and the yellow precipitate was filtered off and washed with water (15 mL) and ethanol (10 mL), to yield the crude intermediate **14** as a orange powder (910 mg; 81%).

Intermediate **14** (50 mg; 0.188 mmol) was suspended in ethanol (4 mL), and oxygen was bubbled through this mixture at 50°C. Within minutes, the color changed from yellow to red. After 16 hr, the precipitate was filtered off, and washed with ethanol, to give the product **15** as a red powder (31 mg, 62%).

^{1}H NMR (DMSO-d_6): δ = 7.77 (s, 2H), 7.66 (d, 2H), 7.30 (t, 2H), 6.85 (d, 2H), 5.53 (s, 4H) ppm. HPLC-MS/PDA: one peak in chromatogram, m/z = 265.2 ($M+H^+$), λ_{max} = 240, 296 and 527 nm.

*Synthesis of 3,6-bis(aminomethyl)-1,2,4,5-tetrazine (**20**)*



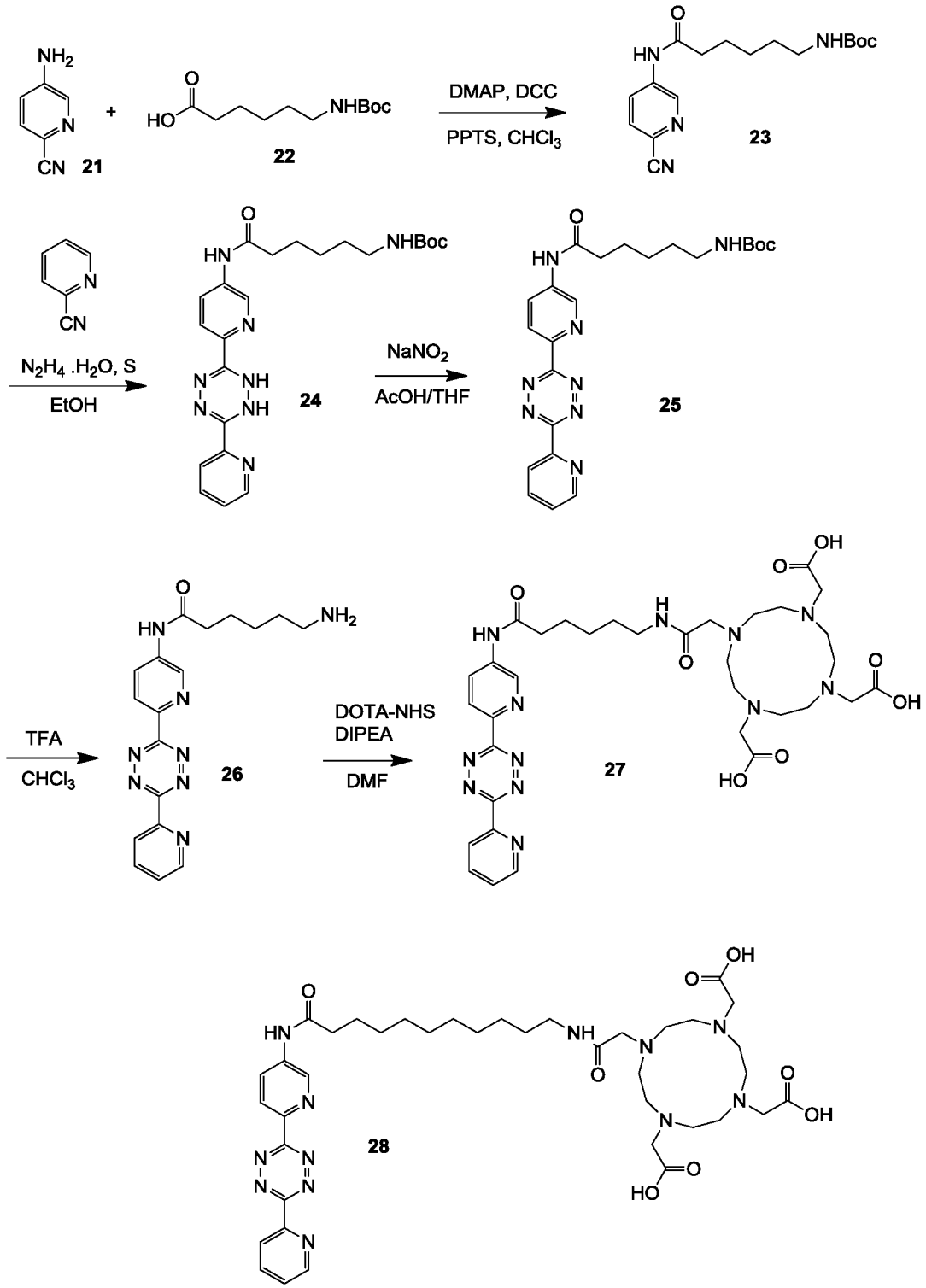
Boc-amino acetonitrile (1.00 g; 6.40 mmol) was dissolved in methanol (10 mL) and sodium methoxide (0.145 mL 25% in MeOH; 0.64 mmol) was added. The mixture was stirred at 20°C for 18 hr, and subsequently ammonium chloride (0.34 g; 6.40 mmol) was added, and the mixture was stirred at 20°C for 3 days. The solution was precipitated in diethyl ether (40 mL), and the precipitate was collected by filtration, washed, and dried to yield the amidine hydrochloride **17**.

The amidine hydrochloride (**17**, 241 mg; 1.15 mmol) was dissolved in hydrazine hydrate (3 mL; 61.9 mmol), and the solution was stirred at 20°C for 16 hr. Then it was diluted with water (10 mL), and the precipitate was collected by centrifugation, and dried. The colorless solid was dissolved in acetic acid (1.5 mL) and sodium nitrite (28 mg; 0.41 mmol) was added. The pink mixture was stirred for 15 min and subsequently chloroform (15 mL) and saturated sodium bicarbonate (30 mL) were added. The organic layer was isolated and washed with water (15 mL), dried over sodium sulfate, and evaporated to dryness, to yield the Boc-protected tetrazine as a pink solid (**19**, 70 mg; 35%). This compound (12 mg; 0.035 mmol) was dissolved in chloroform (1 mL), and TFA (1 mL) was added. The mixture was stirred for 15 min, and the precipitated in diethyl ether (15 mL). The pink precipitate was filtered off, washed, and dried to give the title compound as its TFA salt (**20**, 10 mg, 78%).

^{1}H NMR (D_2O): δ = 5.06 (s, 4H) ppm. ^{13}C NMR (D_2O): δ = 164.5, 41.1 ppm. HPLC-MS/PDA: one peak in chromatogram, m/z = 141 ($M+H^+$), λ_{max} = 267 and 517 nm.

*Synthesis of 2,2',2''-(10-(2-oxo-2-(6-oxo-6-(6-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-ylamino)hexylamino)ethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid (**27**) and 2,2',2''-(10-(2-oxo-2-(11-oxo-11-(6-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-ylamino)undecylamino)ethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid (**28**)*

2012257418 29 Jun 2017



5-Amino-2-cyanopyridine **21** (1.02 g; 8.60 mmol), N-Boc-6-amino-hexanoic acid **22** (0.99 g; 4.30 mmol), DCC (1.77 g; 8.60 mmol), DMAP (1.05 g; 8.60 mmol), and PPTS (0.37 g; 1.47 mmol) were suspended in chloroform (15 mL). The mixture was stirred at room temperature for 18 hr, and then evaporated to dryness, and stirred in acetonitrile (20 mL). The precipitate was removed by filtration, and the filtrate was evaporated to dryness,

dissolved in chloroform (20 mL), and washed with respectively aqueous citric acid (15 mL 0.5 M), aqueous potassium hydrogencarbonate (15 mL, 1 M), and water (15 mL). The organic phase was dried over sodium sulfate and evaporated to dryness. The crude product was purified by column chromatography (silica, hexane/ethylacetate=1:1) to yield the product **23** as a white solid (0.95 g; 61%).

MS (ESI, *m/z*): Calcd for $C_{17}H_{25}N_4O_3^+$ ($[M+H]^+$): 333.19, Found: 333.17.

Tert-butyl 6-(6-cyanopyridin-3-ylamino)-6-oxohexylcarbamate **23** (0.70 g; 2.1 mmol), 2-cyanopyridine (0.87 g; 8.4 mmol), hydrazine hydrate (1.25 g; 20 mmol) were dissolved in ethanol (2 mL), and sulfur (0.22 g; 7 mmol) was added. The mixture was stirred at 70°C under an inert atmosphere of argon for 2 hr, and then at 50°C for 16 hr. The orange suspension was diluted with chloroform (10 mL), and the resulting solution was washed with water (2 times 15 mL). The organic phase was dried over sodium sulfate and evaporated to dryness. The crude product was purified by column chromatography (silica, chloroform/acetone=4:1) to yield the product **24** as an orange solid (0.65 g; 66%). MS (ESI, *m/z*): Calcd for $C_{23}H_{31}N_8O_3^+$ ($[M+H]^+$): 467.25, Found: 467.33.

Tert-butyl 6-oxo-6-(6-(6-(pyridin-2-yl)-1,2-dihydro-1,2,4,5-tetrazin-3-yl)pyridin-3-ylamino)hexylcarbamate **24** (0.30 g; 0.64 mmol) was dissolved in THF (1.5 mL), and acetic acid (2 mL) was added. Sodium nitrite (0.25 g; 3.62 mmol) was dissolved in water (1 mL) and added dropwise. The red solution was poured in aqueous potassium hydrogencarbonate (50 mL; 1 M), and the product was extracted with chloroform (50 mL). The organic layer was washed with water (50 mL), and dried over sodium sulfate and evaporated to dryness, to yield the product **25** as a purple solid (0.25 g; 83%).
MS (ESI, *m/z*): Calcd for $C_{23}H_{29}N_8O_3^+$ ($[M+H]^+$): 465.23, Found: 465.42.

tert-Butyl 6-oxo-6-(6-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-ylamino) hexylcarbamate **25** (66 mg; 0.14 mmol) was dissolved in chloroform (6 mL), and TFA (6 mL) was added. The solution was stirred at room temperature for 2 hr, and subsequently evaporated to dryness, to yield the product **26** as its TFA salt (52 mg; 100%).
MS (ESI, *m/z*): Calcd for $C_{18}H_{21}N_8O^+$ ($[M+H]^+$): 365.19, Found: 365.33.

6-Amino-N-(6-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-yl)hexanamide **26** (52 mg; 0.14 mmol) was dissolved in DMF (2.5 mL), and DIPEA was added (320 mg; 2.0 mmol). *N*-Hydroxysuccinimide activaded DOTA (161 mg; 0.2 mmol) was added, and the mixture was stirred at room temperature for 5 hr. The solution was evaporated to dryness, and the crude product was dissolved in a mixture of acetonitrile and water, and purified by

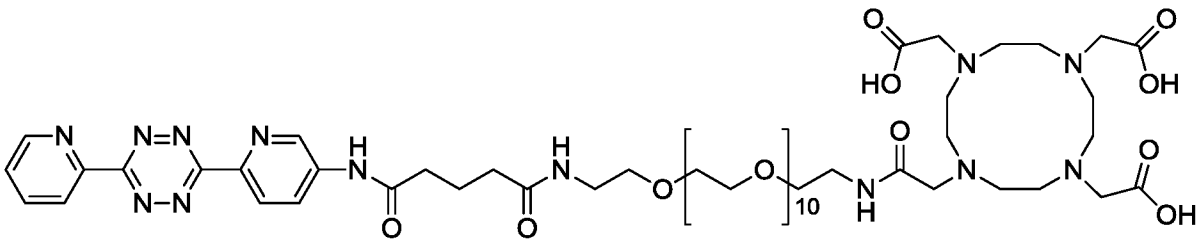
preparative RP-HPLC. After lyophilisation the pure product **27** was obtained as a pink fluffy solid (80 mg, 76% yield).

^{1}H-NMR (30% acetonitrile-d_3 in D_2O): δ = 8.90 (m, 2H, Ar*H*), 8.68 (d, 1H, Ar*H*), 8.60 (dd, 1H, Ar*H*), 8.31 (m, 1H, Ar*H*), 8.24 (t, 1H, Ar*H*), 7.82 (t, 1H, Ar*H*), 3.80 (br s, 6H, NCH_2COOH), 3.72 (br s, 2H, NCH_2CONH), 3.34-3.23 (br m, 18H, NCH_2CH_2N, CH_2NHCO), 2.49 (t, 2H, NHCOCH_2), 1.70 (m, 2H, NHCOCH$_2$CH_2), 1.59 (m, 2H, CH_2CH$_2$NHCO), 1.41 (m, 2H, CH_2CH$_2$CH$_2$NHCO) ppm. ^{13}C-NMR (30% acetonitrile-d_3 in D_2O): δ = 175.5, 171.5 (br), 162.6, 162.5, 150.1, 148.1, 142.9, 141.6, 139.6, 138.4, 128.0, 127.9, 125.4, 124.8, 55.4, 54.3 (br), 49.4 (br), 39.4, 36.5, 28.2, 25.9, 24.6 ppm. ESI-MS: *m/z* for $C_{34}H_{47}N_{12}O_8^+$ ([*M*+H]$^+$): 751.37; Obs. [M+H]$^+$ 751.58, [M+Na]$^+$ 773.50, [M+2H]$^{2+}$ 376.42, [M+3H]$^{3+}$ 251.33. FT-IR (ATR): υ = 3263, 3094, 2941, 2862, 1667, 1637, 1582, 1540, 1460, 1431, 1395, 1324, 1296, 1272, 1251, 1226, 1198, 1128, 1087, 1060, 1020, 992, 977, 920, 860, 831, 798, 782, 742, 718, 679, 663 cm^{-1}.

For **28**, a procedure was used comparable to the described synthesis of 2,2',2''-(10-(2-oxo-2-(6-oxo-6-(6-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-ylamino) hexylamino)ethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid (**27**).
After lyophilisation the pure product **28** was obtained as a pink fluffy solid (90 mg, 78% yield).

^{1}H-NMR (DMSO-d_6): δ = 10.65 (s, 1H, N*H*), 9.06 (d, 1H, Ar*H*), 8.93 (d, 1H, Ar*H*), 8.61 (t, 2H, Ar*H*), 8.44 (dd, 1H, Ar*H*), 8.16 (t, 2H, Ar*H*, N*H*), 7.73 (dd, 1H, Ar*H*), 3.51 (br s, 6H, NCH_2COOH), 3.28 (br s, 2H, NCH_2CONH), 3.06 (q, 2H, CH_2NHCO), 3.34-3.23 (br m, 16H, NCH_2CH_2N), 2.43 (t, 2H, NHCOCH_2), 1.64 (m, 2H, NHCOCH$_2$CH_2), 1.42 (m, 2H, CH_2CH$_2$NHCO), 1.38-1.22 (m, 12H, CH_2) ppm. ^{13}C-NMR (DMSO-d_6): δ = 173.0, 171.0 (br), 169.1 (br), 163.5, 163.2, 151.0, 150.6, 144.2, 141.7, 139.1, 138.2, 127.0, 126.5, 125.3, 124.6, 57.3 (br), 55.2 (br), 50.7, 39.0, 36.8, 29.5, 29.4, 29.3, 29.19, 29.17, 29.1, 26.9, 25.3 ppm. ESI-MS: *m/z* Calcd for $C_{39}H_{57}N_{12}O_8^+$ ([*M*+H]$^+$): 821.44; Obs. [M+Na]$^+$ 843.58, [M+H]$^+$ 821.58, [M+2H]$^{2+}$ 411.42, [M+3H]$^{3+}$ 274.67. FT-IR (ATR): υ = 3261, 3067, 2925, 2851, 1633, 1583, 1541, 1458, 1433, 1394, 1324, 1298, 1270, 1249, 1228, 1200, 1165, 1128, 1088, 1059, 1016, 991, 920, 885, 860, 832, 798, 782, 764, 742, 719, 687, 661 cm^{-1}.

*DOTA-tetrazine Activator **29***



29

The tetrazine **29** above has been described in detail in Robillard et al., Angew. Chem., 2010, 122, 3447-3450. It also serves as an example a structure that can be used as an Activator according to this invention. The amide function on one of the 2-pyridyl groups of the 1,2,4,5-tetrazine moiety is an electron donating group, while both pyridine groups can be viewed as electron withdrawing. The tetrazine can therefore be seen as slightly electron deficient.

Activator **29** displays suitable and favorable pharmacological properties: **29** is rather stable in PBS solution with little degradation within 2 hrs and most of the material still intact after overnight incubation; it has a 10 min blood clearance half-life in mice; its partial volume of distribution (V_d) in mice corresponds to the total extracellular water compartment, as it does not significantly enter cells. Activator **29** contains a DOTA ligand, and such ligands are instrumental in a variety of imaging modalities (e.g. MRI, SPECT, PET). Consequently, Activator **29** is not only suitable for drug release, but it can simultaneously be used for imaging purposes. In fact, Activator **29** has been employed as a SPECT/CT imaging probe after complexation with $^{111}In^{3+}$. See Robillard et al., Angew. Chem., 2010, 122, 3447-3450 for further details.

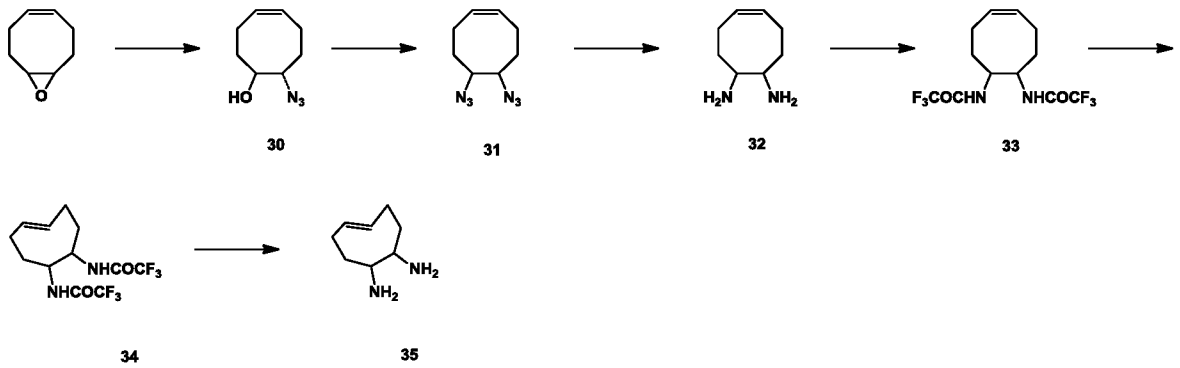
Note that the amino-1,2,4,5-tetrazine moieties comprised in Activators **27** – **29** can be used for conjugation to a range additional functional groups such as sugars, PEG, polymers, peptides (such as RGD or c-RGD), proteins, fluorescent molecules or dye molecules.

Example 2

Synthesis of (*E*)-cyclooctene model prodrugs and prodrugs

*Synthesis cis-(E)-cyclooct-5-ene-1,2-diamine (**35**)*

2012257418 29 Jun 2017



Epoxycyclooctene was prepared by reaction of 1,5-cyclooctadiene with sodium perborate in acetic acid and dichloromethane, cf. Günes, Y.; Senocak, E.; Tosun, C.; Taskesenligil, Y., Org. Commun. 2009, 2:3, 79-83. The crude product was used as such. A solution of epoxycyclooctene (80.0 g, 0.645 mol) in 140 mL acetone was added over a 45 min period to a solution of sodium azide (80 g, 1.23 mol) in 200 mL water. The addition funnel was flushed with 20 mL acetone and the mixture was heated under reflux for 76 hrs. Most of the acetone was removed by rotary evaporation, 100 mL water was added to the residue and the mixture was extracted with 3 x 250 mL TBME. The organic layers were washed with 100 mL water, then dried and rotary evaporated to yield crude *trans-(Z)*-8-azidocyclooct-4-enol (**30**) (mixed with the epoxide) which was used as such in the next step. ^{1}H-NMR ($CDCl_3$): δ = 1.6 – 2.6 (m, 8H), 3.65 – 3.8 (m, 2H), 5.5 – 5.65 (m, 2H) ppm.

Toluene (200 mL) was added to all of **30** and about 150 mL of solvent was removed by rotary evaporation. 300 mL toluene was added to the remainder (a ca. 1/1 mixture of the azido alcohol **30** and the starting epoxide) and the solution was cooled in ice. Triethylamine (86.1 g, 0.852 mol) was added, followed by the addition of methanesulfonyl chloride (93.8 g, 0.819 mol) in 100 mL toluene over a 1 hr period and with mechanical stirring. The suspension was stirred for 2 days, then 200 mL water was added. The layers were separated and the organic layer was washed with 2 x 50 mL water. The successive aqueous layers were extracted with 250 mL toluene. Drying and rotary evaporation yielded a residue which was a mixture of the azido mesylate and the starting epoxide. ^{1}H-NMR ($CDCl_3$): δ = 1.95 – 2.6 (m, 8H), 3.95 (dt, 1H), 4.8 (dt, 1H), 5.5 – 5.65 (m, 2H) ppm.

Half of the residue was warmed for 44 hrs at 75^{0}C with 100 mL DMF and sodium azide (20 g, 0.307 mol), then for 3 hrs at 85^{0}C. The mixture was poured into 200 mL water and then extracted with 3 x 200 mL TBME. The organic layers were washed with 3 x 50 mL water, then dried and rotary evaporated to yield a residue which was a mixture of the *cis-(Z)*-5,6-diazidocyclooct-1-ene (**31**), epoxycyclooctene and impurities.

^{1}H-NMR ($CDCl_3$): δ = 1.8 (m, 2H), 1.95 – 2.1 (m, 4H), 2.5 – 2.65 (m, 2H), 3.8 (dt, 2H), 5.6 – 5.7 (m, 2H) ppm.

The crude diazide **31** obtained above was dissolved in 150 mL THF and added over a 90 min period to lithium aluminium hydride (12.0 g, 0.315 mol) in 200 mL THF, cooling being done with cold water. The reaction mixture was heated under reflux for 8 hrs, then it was cooled and slowly quenched with 6 mL water and 12 mL 30% sodium hydroxide solution. Filtration, washing with THF and rotary evaporation yielded a residue (**32**), which was dissolved in 150 mL dichloromethane, then cooled in ice. Trifluoroacetic anhydride (69.0 g, 0.328 mol) was added over a 30 min period. The solution was stirred for 4 hrs then rotary evaporated. The residue was chromatographed on a 250 g silicagel column, elution being performed with heptane containing increasing amounts of ethyl acetate. The first fractions were the trifluoroacetate of cyclooct-2-en-1-ol. The fractions with the desired *cis*-(*Z*)-N,N'-(cyclooct-5-ene-1,2-diyl)bis(2,2,2-trifluoroacetamide) (**33**) were combined and recrystallized from a mixture of TBME and heptane to give 18.85 g of the product (**33**, 56.74 mmol, 18% based on epoxycyclooctene).

^{1}H-NMR of diamine **32** ($CDCl_3$): δ = 1.65 (m, 2H), 1.8 (m, 2H), 2.0 (m, 2H), 2.4 (m, 2H), 3.0 (dt, 2H), 5.6 (m, 2H) ppm.

^{1}H-NMR of bisamide **33** ($CDCl_3$): δ = 1.65 (m, 2H), 2.0 (m, 2H), 2.2 (m, 2H), 2.35 (m, 2H), 4.15 (dt, 2H), 5.9 (m, 2H), 7.5 (m, 2H) ppm. ^{13}C-NMR ($CDCl_3$): δ 23 (CH_2), 32 (CH_2), 54 (CH), 110 -122 (q, CF_3), 132 (CH), 157 – 159 (q, C=O) ppm. ^{19}F-NMR ($CDCl_3$): δ = -76 ppm.

The crude trifluoroacetamide **33** obtained after evaporation of the reaction product from 3.50 g (25.0 mmol) *cis*-(*Z*)-cyclooct-5-ene-1,2-diamine **32** and trifluoroacetic anhydride (12.3 g, 58.6 mmol) was mixed with 4.0 g methyl benzoate and ca. 500 mL heptane / ether (ca. 2:1). The mixture was irradiated for 42 hrs while the solution was continuously flushed through a 41 g silver nitrate impregnated silicagel column (containing ca. 4.1 g silver nitrate). The column was flushed with 150 mL TBME, then with 150 mL TBME containing some methanol. The fractions were washed with 100 mL 15% ammonia, dried and rotary evaporated. The first fraction yielded a 1:2 mixture of the Z and E alkene, the second fraction yielded a small amount of the E alkene. The column material was stirred with TBME and ammonia, then filtered and the layers were separated. The solid was treated once more with the aqueous layer and TBME, then filtered and the layers were separated. The organic layers were dried and rotary evaporated to yield 3.07 g of the E alkene *cis-(E)-*

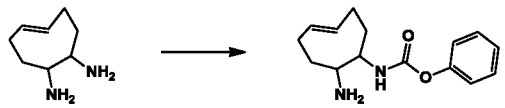
N,N'-(cyclooct-5-ene-1,2-diyl)bis(2,2,2-trifluoroacetamide) (**34**, 9.25 mmol, 37% based on the amine).

^{1}H-NMR ($CDCl_3$): δ = 1.6 - 1.9 (m, 4H), 2.1 - 2.5 (m, 4H), 3.8 (m, 1H), 4.1 (t, 1H), 5.4 – 5.55 (m, 1H), 5.65 – 5.8 (m, 1H), 6.4 (bs, 1H), 7.9 (bs, 1H) ppm.

The amide **34** obtained above was mixed with 40 mL methanol, 5.0 g sodium hydroxide and 10 mL water. The mixture was warmed for 90 min at near reflux, then it was rotary evaporated and the residue was diluted with 30 mL water. Extraction with 3 x 50 mL dichloromethane, drying and rotary evaporation yielded the desired diamine *cis-(E)*-cyclooct-5-ene-1,2-diamine (**35**), containing a small amount of solvent (1.38 g, ca. 100%).

^{1}H-NMR ($CDCl_3$): δ = 1.4 – 2.5 (m, 8H), 2.8 (bs, 1H), 2.9 (d, 1H), 5.4 – 5.6 (m, 2H) ppm.

Synthesis of cis-*(E)-phenyl (8-aminocyclooct-4-en-1-yl)carbamate (**36**)*

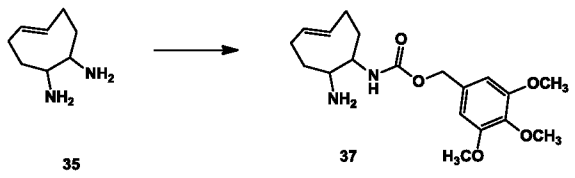


35 **36**

Diphenylcarbonate (500 mg, 2.33 mmol) was added to a solution of the diamine **35** (300 mg, 2.14 mmol) in 10 mL dichloromethane and the solution was stirred for 4 days at room temperature. The solution was chromatographed on 25 g silica, eluting with dichloromethane containing increasing amounts of methanol. The product fractions were combined and stirred with 15 mL TBME for 2 hrs. 15 mL heptane was added and the mixture was filtered. The solid was stirred with 15 mL TBME then filtered. The combined filtrates were rotary evaporated and the residue was stirred overnight with heptane to give a solid. Filtration yielded the desired product *cis*-(E)-phenyl (8-aminocyclooct-4-en-1-yl)carbamate (**36**).

^{1}H-NMR ($CDCl_3$): δ = 1.5 (bs, 4H), 1.8 – 2.35 (m, 6H), 3.1 (bs, 1H), 3.6 (t, 1H), 5.5 (bd, 1H), 5.6 (m, 2H), 7.05 – 7.4 (m, 5H) ppm. ^{13}C-NMR ($CDCl_3$): δ = 28.4 (CH_2), 33.0 (CH_2), 36.7 (CH_2), 40.7 (CH_2), 57.7 (CH), 58.4 (CH), 121.8 (CH), 125.4 (CH), 129.5 (CH), 133.2 (CH), 133.3 (CH), 151.3 (C), 154.0 (C) ppm.

Synthesis of cis-*(E)-3,4,5-trimethoxybenzyl (8-aminocyclooct-4-en-1-yl)carbamate (**37**)*



A solution of 3,4,5-trimethoxybenzyl alcohol (6.20 g, 31.3 mmol) in THF (20 mL) was added in 15 min to a solution of CDI (5.44 g, 33.58 mmol) in THF (25 mL), cooled in an ice-water bath. The mixture was then stirred for 3 days at room temperature. The solvent was removed under vacuum and TBME (100 mL) was added. The mixture was stirred for 1 hr, decanted and filtered. The residue was stirred with TBME (25 mL) for 5 min, decanted and filtered. The combined TBME filtrates were concentrated to give the CDI-adduct (10.45 g, 35.78 mmol, not corrected for free imidazole) as an oil which slowly solidified. By NMR it contains one equivalent of free imidazole.

^{1}H-NMR ($CDCl_3$): δ = 3.85 (s, 3H), 3.9 (s, 6H), 5.35 (s, 2H), 6.65 (s, 2H), 7.05 (s, 1H), 7.45 (s, 1H), 8.2 (s, 1H) ppm.

The CDI-derivative (705 mg, 2.41 mmol) was added to a solution of the diamine **35** (330 mg, 2.35 mmol) in dichloromethane (15 mL) and the mixture was stirred for 1 hr at room temperature. The mixture was concentrated, ethyl acetate (20 mL) was added to the residue and the mixture was filtered while warm. The residue was washed with warm ethyl acetate and the combined filtrates were cooled in ice until a precipitate started to appear, then it was stirred for 1 hr. The mixture was cooled for 30 min at – 15 °C and the precipitate collected by filtration, washed with cold ethyl acetate and dried to give *cis*-(*E*)-3,4,5-trimethoxybenzyl (8-aminocyclooct-4-en-1-yl)carbamate **37** (170 mg, 0.47 mmol, 20%).

^{1}H-NMR ($CDCl_3$): δ = 1.4 – 2.3 (m, 10H), 3.05 (bs, 1H), 3.55 (t, 1H), 3.8 (s, 3H), 3.85 (s, 6H), 5.0 (s, 2H), 5.2 (m, 1H), 5.6 (m, 2H), 6.6 (s, 2H), 7.45 (s, 1H), 8.2 (s, 1H) ppm. ^{13}C-NMR ($CDCl_3$): δ = 28 (CH_2), 33 (CH_2), 37 (CH_2), 41 (CH_2), 56 (CH), 58 (CH), 58.5 (CH), 61 (CH), 67 (CH_2), 106 (CH), 132.5 (C), 133 (CH), 133.5 (CH), 138 (C), 153.5 (C), 155.5 (C) ppm.

Synthesis of cis *2-methylphenyl ((E)-8-aminocyclooct-4-en-1-yl)carbamate (**39**)*

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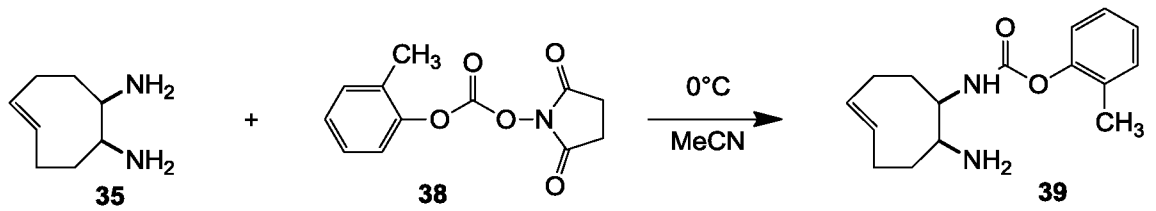
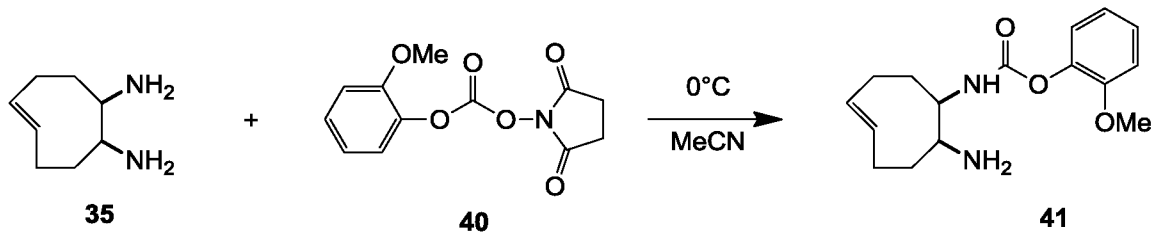


Cis-(*E*)-cyclooct-5-ene-1,2-diamine (**35**, 9.7 mg; 0.069 mmol) was dissolved in acetonitrile (1 mL) and cooled to 0°C. NHS-activated o-cresol **38** (17.2 mg; 0.069 mmol) was dissolved in acetonitrile (1 mL) and was added. The mixture was stirred for 30 min at 0°C. The precipitate was removed by filtration, and the filtrate was evaporated to dryness. The oily residue was dissolved in dichloromethane (2 mL) and washed with water (1 mL). The product was extracted with 0.5 M citric acid (1.5 mL) and the aqueous phase was isolated and neutralized with brine (2 mL). The product was extracted with dichloromethane (two times 2 mL). The combined organic layers were dried over sodium sulfate and evaporated to dryness, to give the product **39** as a viscous oil (6.1 mg, 32%).

^{1}H NMR ($CDCl_3$): δ = 7.2 – 7.0 (m, 4H), 5.62 (m, 2H), 5.57 (s, 1H), 3.59 (t, 1H), 3.14 (d, 1H), 2.19 (s, 3H), 2.3 – 1.8 (m, 8H), 1.6 (br. s, 2H) ppm. HPLC-MS/PDA: one peak in chromatogram, m/z = 275 ($M+H^+$), λ_{max} = 261 nm.

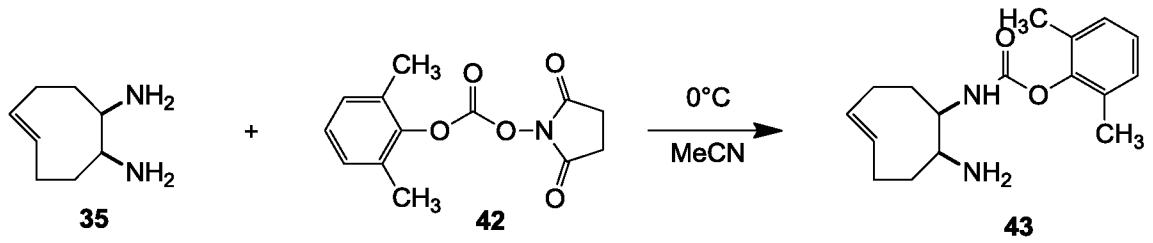
Synthesis of cis *2-methoxyphenyl ((E)-8-aminocyclooct-4-en-1-yl)carbamate (**41**)*



Cis-(*E*)-cyclooct-5-ene-1,2-diamine (**35**, 9.7 mg; 0.069 mmol) was dissolved in acetonitrile (1 mL) and cooled to 0°C. NHS-activated 2-methoxyphenol (**40**, 18.3 mg; 0.069 mmol) was dissolved in acetonitrile (1 mL) and was added. The mixture was stirred for 45 min at 0°C. The precipitate was removed by filtration, and the filtrate was evaporated to dryness. The oily residue was dissolved in dichloromethane (2 mL) and washed with water (1 mL). The product was extracted with 0.5 M citric acid (1.5 mL) and the aqueous phase was isolated and neutralized with brine (2 mL). The product was extracted with dichloromethane (two times 2 mL). The combined organic layers were dried over sodium sulfate and evaporated to dryness, to give the product **41** as a viscous oil (9.0 mg, 45%).

^{1}H NMR ($CDCl_3$): δ = 7.22 (m, 2H), 6.97 (m, 2H), 5.62 (m, 2H), 5.71 (s, 1H), 3.89 (s, 3H), 3.59 (t, 1H), 3.14 (d, 1H), 2.3 – 1.8 (m, 8H), 1.6 (br. s, 2H) ppm. HPLC-MS/PDA: one peak in chromatogram, m/z = 291 ($M+H^+$), λ_{max} = 269 nm.

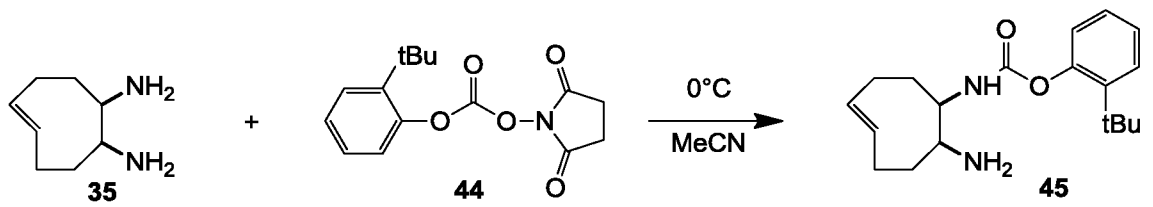
Synthesis of cis *2,6-dimethylphenyl ((E)-8-aminocyclooct-4-en-1-yl)carbamate (**43**)*



Cis-(*E*)-cyclooct-5-ene-1,2-diamine (**35**, 26.5 mg; 0.189 mmol) was dissolved in acetonitrile (1.5 mL) and cooled to 0°C. NHS-activated 2,6-dimethylphenol (**42**, 50 mg; 0.189 mmol) was added, and the mixture was stirred for 1 hr. The precipitate was removed by filtration, and the filtrate was evaporated to dryness. The oily residue was dissolved in dichloromethane (3 mL) and washed with water (1.5 mL). The product was extracted with 0.5 M citric acid (2 mL) and the aqueous phase was isolated and neutralized with brine (3 mL). The product was extracted with dichloromethane (two times 3 mL). The combined organic layers were dried over sodium sulfate and evaporated to dryness, to give the product **43** as a viscous oil (32 mg, 60%).

^{1}H NMR ($CDCl_3$): δ = 7.02 (m, 3H), 5.57 (m, 2H), 5.49 (s, 1H), 3.58 (t, 1H), 3.10 (d, 1H), 2.17 (s, 6H), 2.3 – 1.8 (m, 8H), 1.6 (br. s, 2H) ppm. HPLC-MS/PDA: one peak in chromatogram, m/z = 289 ($M+H^+$), λ_{max} = 259 nm.

Synthesis of cis *2-tert-butylphenyl ((E)-8-aminocyclooct-4-en-1-yl)carbamate (**45**)*

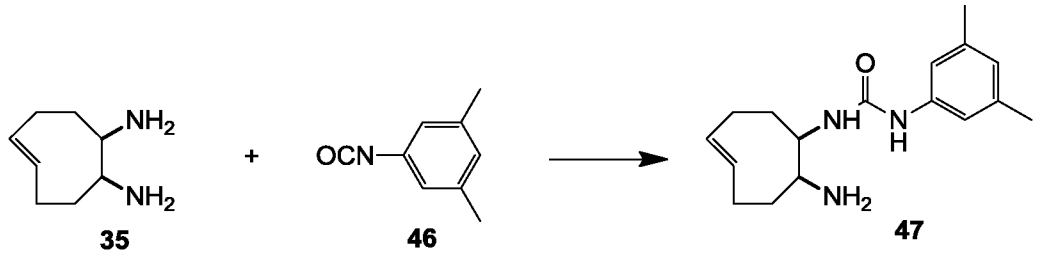


Cis-(*E*)-cyclooct-5-ene-1,2-diamine (**35**, 26.5 mg; 0.189 mmol) was dissolved in acetonitrile (1.5 mL) and cooled to 0°C. NHS-activated 2-*tert*-butylphenol (**44**, 55 mg; 0.189 mmol) was added, and the mixture was stirred for 1 hr, and then evaporated to dryness. The oily residue was dissolved in dichloromethane (3 mL) and washed with water (1.5 mL).

The product was extracted with 0.5 M citric acid (2 mL) and the aqueous phase was isolated and neutralized with brine (3 mL). The product was extracted with dichloromethane (two times 3 mL). The combined organic layers were dried over sodium sulfate and evaporated to dryness, to give the product **45** as a viscous oil (15 mg, 25%).

HPLC-MS/PDA: one peak in chromatogram, m/z =317 ($M+H^+$), λ_{max} = 259 nm.

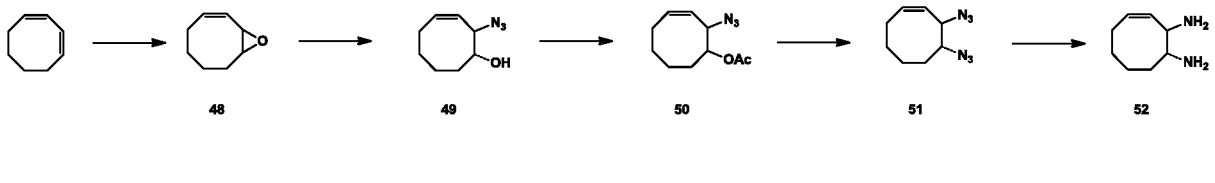
Synthesis of cis *phenyl ((E)-8-aminocyclooct-4-en-1-yl)urea (47)*

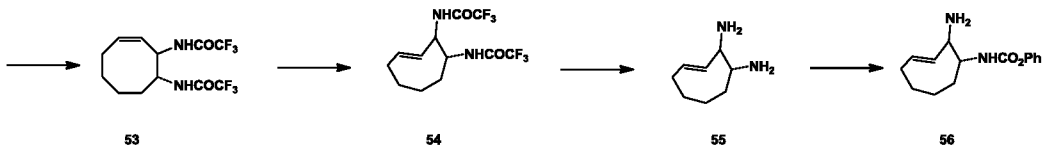


35 **46** **47**

Cis-(*E*)-cyclooct-5-ene-1,2-diamine (**35**, 23.4 mg; 0.167 mmol) was dissolved in chloroform (1 mL). 3,5-Dimethylphenylisocyanate (**46**, 24.5 mg; 0.167 mmol) was dissolved in chloroform (1 mL) and was added slowly at 20°C. The mixture was stirred for 30 min at 20°C, and subsequently concentrated *in vacuo*. The crude material was subsequently purified by prep-HPLC, yielding two isomeric products **47** with m/z =288 ($M+H^+$), λ_{max} = 243 nm: 10.9 mg of major isomer (23% yield) and 1.9 mg of minor isomer (4% yield).

Synthesis of trans *phenyl (E)-2-aminocyclooct-3-en-1-yl carbamate (**56**)*





A mixture of 1,3-cyclooctadiene (21.72 g, 0.201 mol), 200 mL dichloromethane, 75 mL acetic acid and 35.16 g sodium perborate tetrahydrate (0.228 mol) was stirred for 2 days at room temperature, then for 28 hrs at 35°C. The mixture was poured into 150 mL water and 200 mL dichloromethane. The layers were separated and the organic

layer was washed with 50 mL water and with 100 mL 20% sodium hydroxide solution. The successive aqueous layers were extracted with 200 mL dichloromethane (filtration over Celite being necessary). The organic layers were dried and rotary evaporated. The residue comprising (*Z*)-9-oxabicyclo[6.1.0]non-2-ene (**48**) was used as such.

A solution of the epoxycyclooctene **48** obtained above in 65 mL acetone was added over a 30 min period to a solution of sodium azide (24.0 g, 0.369 mol) in 60 mL water. The mixture was heated under reflux for 7 days, distilling off ca. 30 mL acetone after 4 days (Note). 50 mL water was added to the residue and the mixture was extracted with 2 x 200 mL TBME. The organic layers were washed with 25 mL water, then dried and rotary evaporated to yield 19.6 g of residue comprising *trans*-(*Z*)-2-azidocyclooct-3-enol (**49**)**.** Note: The ring opening with sodium azide was described in Organic Synthesis 2010, **87**, 161 for cyclohexene oxide. We have our doubts about the reported use of acetone as the organic solvent, because the Organic Synthesis procedure reports a reflux temperature of 85°C, which seems to correspond better with acetonitrile as solvent than acetone.

^{1}H-NMR ($CDCl_3$): δ = 1.2 – 2.4 (m, 8H), 3.55 (m, 1H), 4.35 (t, 1H), 5.55 (m, 1H), 5.85 (m, 1H) ppm.

The crude **49** obtained above was dissolved in 130 mL TBME and 28 mL triethylamine (0.202 mol). The solution was cooled with ethanol – dry ice and methanesulfonyl chloride (21.2 g, 0.185 mol) in 30 mL TBME was added over a 30 min period at -10 to 0°C. The resulting suspension was stirred overnight, then 100 mL water was added. The layers were separated and the organic layer was washed with 50 mL water. The successive aqueous layers were extracted with 200 mL TBME. Drying and rotary evaporation yielded 20.3 g azidomesylate residue which was dissolved in 75 mL DMF. Potassium acetate (19.65 g, 0.20 mol) was added and the mixture was heated for 2 hrs at 80°C. Another 50 mL DMF was added and heating was continued for 17 hrs at 90°C. After cooling, the mixture was poured into 150 mL diluted ammonia and the product was extracted with 3 x 200 mL TBME. The successive organic layers were washed with 3 x 25 mL water, then dried and rotary evaporated. The residue was chromatographed on 150 g silica, elution being done with heptane / ethyl acetate, yielding *cis* (*Z*)-2-azidocyclooct-3-en-1-yl acetate (**50**).

^{1}H-NMR of the azidomesylate ($CDCl_3$): δ = 1.2 – 2.4 (m, 8H), 3.1 (s, 3H), 4.5 – 4.65 (m, 2H), 5.4 (m, 1H), 6.0 (m, 1H) ppm.

^{1}H-NMR of the azido acetate **50** ($CDCl_3$): δ = 1.2 – 2.0 (m, 8H), 2.05 (s, 3H), 4.35 (m, 1H), 5.45 – 5.7 (m, 3H) ppm.

The azidoacetate **50** was stirred for 1 hr with 50 mL methanol and 15 mL 30% sodium hydroxide solution. Most of the methanol was then removed by rotary evaporation. The residue was extracted with 2 x 100 mL TBME. The organic layers were dried and rotary evaporated to leave 10.5 g of azidoalcohol.

This residue was dissolved in 100 mL TBME and triethylamine (21 mL, 0.151 mol) was added. The mixture was cooled in ice and methanesulfonyl chloride (13.75 g, 0.120 mol) in 50 mL TBME was added over a 1 hr period. The resulting suspension was stirred overnight then 100 mL water was added. The layers were separated and the organic layer was washed with 50 mL water. The successive aqueous layers were extracted with 100 mL TBME. Drying and rotary evaporation yielded the azidomesylate derivative which was dissolved in 45 mL DMF. Sodium azide (11.0 g, 0.169 mol) was added and the mixture was heated for 18 hrs at 70°C, then for 3 hrs at 90°C. After cooling, the mixture was poured into 150 mL water and the product was extracted with 3 x 150 mL TBME. The successive organic layers were washed with 2 x 50 mL water, then dried and rotary evaporated. The residue comprising *trans*-(*Z*)-3,4-diazidocyclooct-1-ene (**51**, 11.28 g) was used as such in the next step.

^{1}H-NMR of the azido alcohol ($CDCl_3$): δ = 1.2 – 2.2 (m, 8H), 4.25 (m, 1H), 4.55 (m, 1H), 5.45 (m, 1H), 5.7 (m, 1H) ppm.

^{1}H-NMR of the azidomesylate ($CDCl_3$): δ = 1.2 – 2.3 (m, 8H), 3.0 (s, 3H), 4.25 (m, 1H), 5.4 (m, 1H), 5.55 – 5.8 (m, 1H) ppm.

^{1}H-NMR of the diazide **51** ($CDCl_3$): δ = 1.2 – 2.4 (m, 8H), 3.45 (m, 1H), 4.35 (m, 1H), 5.45 (m, 1H), 5.95 (m, 1H) ppm.

The crude diazide **51** obtained above was dissolved in 100 mL THF and added over a 30 min period to lithium aluminium hydride (5.4 g, 0.142 mol) in 100 mL THF, cooling being done with cold water. The reaction mixture was heated under reflux for 18 hrs, then it was cooled and slowly quenched with 6 mL water and 6 mL 30% sodium hydroxide solution.

Filtration, washing with THF and rotary evaporation yielded 7.8 g crude diamine **52** which was dissolved in 100 mL dichloromethane, then cooled in ice. Trifluoroacetic anhydride (20.87 g, 0.099 mol) was added over a 30 min period. The solution was stirred for 30 min, heated under reflux for 1 hr, and rotary evaporated. The residue was chromatographed on 100 g silica, elution being performed with heptane containing increasing amounts of ethyl acetate. The product fractions were combined and the residue was stirred

with a mixture of TBME and heptanes to give a suspension. Filtration yielded 5.86 g of *trans* N,N'-((*Z*)-cyclooct-3-ene-1,2-diyl)bis(2,2,2-trifluoroacetamide) (**53**, 17.64 mmol, 9% based on 1,3-cyclooctadiene).

^{1}H-NMR of the diamine **52** ($CDCl_3$): δ = 1.2 – 2.3 (m, 8H), 2.55 (m, 1H), 3.4 (t, 1H), 5.35 (m, 1H), 5.6 (m, 1H) ppm.

^{1}H-NMR of bisamide **53** ($CDCl_3$): δ = 1.3 – 2.0 (m, 6H), 2.25 (m, 2H), 4.05 (m, 1H), 5.0 (q, 1H), 5.4 (t, 1H), 5.9 (q, 1H), 7.0 (bs, 1H), 7.1 (bs, 1H) ppm.

The trifluoroacetamide **53** (5.86 g, 17.64 mmol) was mixed with 6.25 g methyl benzoate and ca. 500 mL heptane / ether (ca. 1:2). The suspension was irradiated for 78 hrs while the mixture was continuously flushed through a 32.2 g silver nitrate impregnated silicagel column (containing ca. 3.2 g silver nitrate). The undissolved amide **53** was collected on top of the column and dissolved very slowly during the irradiation and flushing process and was not yet completely dissolved at the end of the irradiation. The column material was flushed with 300 mL heptane / TBME (1:1), then with 300 mL TBME. The fractions were washed with 100 mL 15% ammonia, dried and rotary evaporated, affording a mixture of **53** and **54** from which **54** could be purified by stirring with heptane : TBME. The column material was stirred with dichloromethane and ammonia, then filtered and the layers were separated. The solid was treated once more with the aqueous layer and dichloromethane, then filtered and the layers were separated. The combined organic layers were dried and rotary evaporated to yield the trans alkene **54** (0.91 g, 16%).

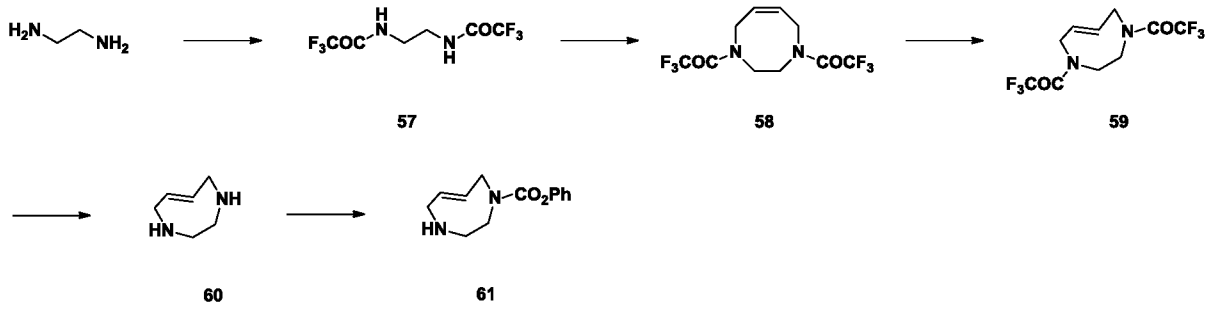
^{1}H-NMR of **54** ($CDCl_3$): δ = 0.8 – 2.5 (m, 8H), 4.35 (m, 1H), 4.55 (m, 1H), 5.7 – 6.0 (m, 2H) ppm. ^{19}F-NMR ($CDCl_3$): δ = -75.9, -76.1 ppm (in addition, there are two small signals at -76.4 and -76.6 ppm, possibly another E-isomer).

Amide **54** (430 mg, 1.29 mmol) was mixed with 10 mL methanol and 1.65 g 50% sodium hydroxide solution was added. The mixture was warmed for 90 min at near reflux, then it was rotary evaporated and the residue was diluted with 15 mL water. Extraction with 4 x 30 mL dichloromethane, drying and rotary evaporation yielded the desired *trans* (*E*)-cyclooct-3-ene-1,2-diamine (**55**, 128 mg, 0.91 mmol, 71%). ^{1}H-NMR ($CDCl_3$): δ = 1.1 – 2.1 (m, 9H), 2.45 (m, 1H), 3.15 (d, 1H), 3.45 (s, 1H), 5.95 (m, 1H) ppm. ^{13}C-NMR ($CDCl_3$): δ = 20.0 (CH_2), 30.6 (CH_2), 35.9 (CH_2), 36.2 (CH_2), 59.2 (CH), 63.7 (CH), 130. 8 (CH), 133.4 (CH) ppm.

Diphenylcarbonate (200 mg, 0.93 mmol) was added to a solution of the diamine **55** (95 mg, 0.68 mmol) in 10 mL dichloromethane and the solution was stirred for 3 days at room temperature (reaction not yet being complete). The solution was rotary

evaporated and the residue was chromatographed on 13 g silica, eluting with dichloromethane with increasing amounts of methanol. This yielded 52 mg of the desired product *trans*-phenyl (*E*)-2-aminocyclooct-3-en-1-yl)carbamate **56** (0.2 mmol, 30%). A fraction with a slightly lower R_f value was assumed to be the carbamate at the 2-amino position. This product was not obtained in a completely pure form (26 mg, 0.1 mmol, 15%). ^{1}H-NMR ($CDCl_3$): δ = 0.8 – 2.2 (m, 9H), 2.45 (m, 1H), 3.8 – 3.95 (m, 2H), 5.35 (bd, 1H, amide NH), 5.75 (dd, 1H), 6.0 – 6.15 (m, 1H), 7.1 – 7.4 (m, 5H) ppm. ^{13}C-NMR ($CDCl_3$): δ = 21.9 (CH_2), 28.1 (CH_2), 36.1 (CH_2), 36.2 (CH_2), 56.4 (CH), 63.2 (CH), 121.8 (CH), 125.6 (CH), 129.5 (CH), 131.7 (CH), 132.7 (CH), 151.2 (C), 154.3 (C=O) ppm. MS: 261.0 (M+1). The compound which is assumed to be the other isomer has ^{1}H-NMR signals at δ 1.0 – 2.2 (m, 7H), 2.45 (m, 1H), 3.6 (bs, 1H), 3.8 (b, 2H), 4.25 (bs, 1H), 5.6 (bs, 1H), 5.7 (m, 1H), 6.0 (d, 1H), 7.1 – 7.4 (m, 5H) ppm. MS 261.0 (M+1).

*Synthesis of (E)-phenyl 2,3,4,5-tetrahydro-1,4-diazocine-1(8H)-carboxylate (**61**)*



Trifluoroacetic anhydride (92.69 g, 0.441 mol) was added over a 1 hr period to an ice-cooled solution of ethylenediamine (12.09 g, 0.20 mol) in 250 mL dichloromethane. The mixture was warmed to reflux for 1 hr, then rotary evaporated. Water (100 mL) and TBME (250 mL) were added and the mixture was stirred for 1 hr. Filtration and washing with TBME gave the product. The filtrate layers were separated, the organic layer was rotary evaporated and the residue stirred with some TBME. Filtration gave an additional amount of N,N'-(ethane-1,2-diyl)bis(2,2,2-trifluoroacetamide) (**57**) for a total yield of 47.23 g (0.187 mol, 93%).

Product **57** obtained above was stirred for 15 min with 400 mL acetonitrile, 100 g potassium carbonate, and 3.2 g benzyltriethylammonium chloride. Cis-1,4-dichloro-2-butene (26.97 g, 95%, 0.205 mol), dissolved in 50 mL acetonitrile, was added over a 30 min period. The mixture was warmed to 71°C over a 3 hrs period and kept at that temperature for

64 hrs, then heated for 24 hrs at 77°C. The mixture was filtered while warm and the solid was washed with acetonitrile. Rotary evaporation left a residue which was chromatographed on 250 g silica gel using dichloromethane and dichloromethane containing some triethylamine as the eluent. This yielded 23.25 g of (*Z*)-1,1'-(2,3-dihydro-1,4-diazocine-1,4(5H,8H)-diyl)bis(2,2,2-trifluoroethanone) (**58**, 76.43 mmol, 41%).

^{1}H-NMR ($CDCl_3$): δ = 3.65 – 3.95 (m, 5.4H), 4.05 – 4.2 (m, 1.1H), 4.25 (bs, 1.5H), 5.6 (m, 0.8H), 5.8 (bs, 0.5H), 6.15 (m, 0.7H) ppm. ^{13}C-NMR ($CDCl_3$): δ = 45 (2 CH_2), 47 (CH_2), 48 (CH_2), 48.5 (CH_2), 49 (CH_2), 50 (CH_2), 53 (CH_2), 110 -122 (2q, CF_3), 126 (CH), 127 (CH), 128 (CH), 128.5 (CH), 155 – 158 (2q, C=O) ppm. ^{19}F-NMR ($CDCl_3$): δ = -69.2, -69.4, -69.8, -70.0 ppm. MS: 305.0 (M+1), 303.0 (M-1).

The trifluoroacetamide **58** (14.0 g, 46.0 mmol) was mixed with 8.0 g methyl benzoate and ca. 500 mL heptane / ether (ca. 10:1). The mixture was irradiated for 92 hrs while the solution was continuously flushed through a 70 g silver nitrate impregnated silica gel column (containing ca. 7.0 g silver nitrate). The column material was then flushed with 300 mL portions of heptane/TBME in the ratios 5:1, 3:1, 2:1, 1:1 and then with 300 mL TBME, each fraction being washed with 200 mL 10% ammonia, dried and rotary evaporated. The remaining column material was stirred with TBME and ammonia, then filtered and the layers were separated. The solid was treated once more with the aqueous layer and TBME, then filtered and the layers were separated. The combined organic layers were dried and rotary evaporated to yield 3.48 g of (*E*)-1,1'-(2,3-dihydro-1,4-diazocine-1,4(5H,8H)-diyl)bis(2,2,2-trifluoroethanone) (**59**) as a solidifying oil (11.45 mmol, 25%).

^{1}H-NMR ($CDCl_3$): δ = 2.6 (t, 1H), 2.95 (t, 1H), 3.4 -3.55 (m, 1H), 3.75 – 3.9 (m, 1H), 4.0 – 4.3 (m, 1H), 4.35 – 4.6 (m, 1H), 4.65 (d, 1H), 5.25 (d, 1H), 5.8 – 6.0 (m, 2H) ppm. ^{13}C-NMR ($CDCl_3$): δ = 49.4 (CH_2), 49.6 (CH_2), 50.0 (CH_2), 50.1 (CH_2), 51.9 (CH_2), 52.0 (CH_2), 53.9 (CH_2), 54.1 (CH_2), 110 -122 (2q, CF_3), 136.0 (CH), 136.1 (CH), 155 – 158 (2q, C=O) ppm. ^{19}F-NMR ($CDCl_3$): δ = -69.2, -69.25, -69.4, -69.45 ppm.

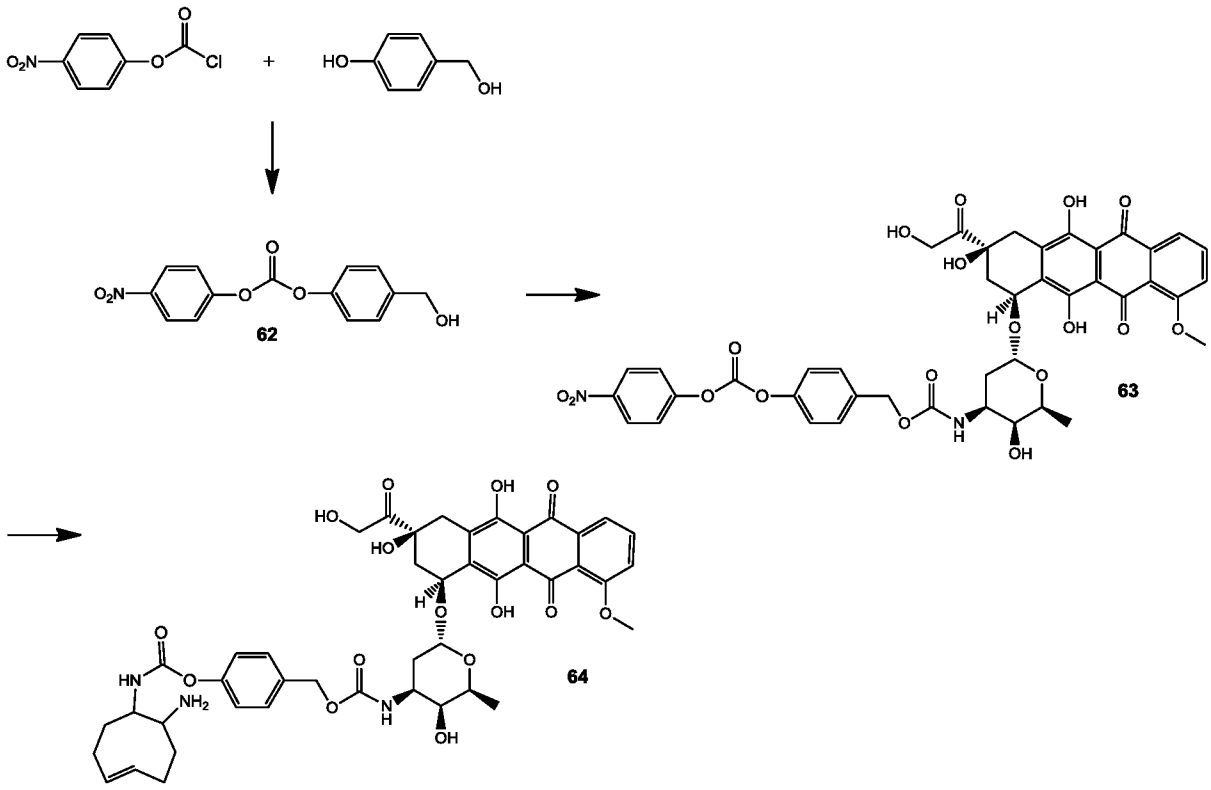
The amide **59** obtained above (520 mg, 1.71 mmol) was mixed with 10 mL methanol and 1.60 g 50% sodium hydroxide solution, then warmed for 1 hr at 55°C. Most of the methanol was removed by rotary evaporation and the residue was diluted with 20 mL water. Extraction with 5 x 25 mL dichloromethane, drying and rotary evaporation yielded the desired (*E*)-1,2,3,4,5,8-hexahydro-1,4-diazocine (**60**, 150 mg, 1.34 mmol, 78%).

^{1}H-NMR ($CDCl_3$): δ = 2.45 (d, 2H), 3.15 (d, 2H), 3.3 (m, 2H), 3.55 (dd, 2H), 6.0 (m, 2H) ppm. ^{13}C-NMR: δ = 53.0 (CH_2), 54.0 (CH_2), 140.0 (CH) ppm.

Diphenylcarbonate (266 mg, 1.24 mmol) was added to a solution of the diamine **60** obtained above in 10 mL dichloromethane and the solution was stirred for 2 days at 30°C. The solution was chromatographed on 17 g silica, eluting with dichloromethane containing increasing amounts of methanol. The product fractions were combined and rotary evaporated. The residue was chromatographed on 20 g silica, eluting with TBME containing increasing amounts of methanol. Further elution with dichloromethane – methanol yielded the product (*E*)-phenyl 2,3,4,5-tetrahydro-1,4-diazocine-1(8H)-carboxylate (**61**).

^{1}H-NMR ($CDCl_3$): δ = 2.5 – 2.85 (m, 2H), 3.25 – 3.4 (m, 2H), 3.4 – 3.55 (m, 2H), 4.25 (m, 1H), 4.9 (m, 1H), 5.8 – 6.1 (m, 2H), 7.0 – 7.4 (m, 5H) ppm. ^{13}C-NMR ($CDCl_3$): δ = 50.2 (CH_2), 50.8 (CH_2), 52.5 (CH_2), 52.6 (CH_2), 53.5 (CH_2), 121.9 (CH), 125.5 (CH), 129.5 (CH), 135.3 (CH), 135.4 (CH), 141.3 (CH), 141.6 (CH), 151.6 (C), 155.0 (C=O) ppm. MS: 232.9 (M+1).

*Synthesis of (*E*)-cyclooctene-doxorubicin conjugate **64***



4-(Hydroxymethyl)phenyl 4-nitrophenyl carbonate (**62**) was synthesized *via* a modified literature procedure (K. Haba, M. Popkov, M. Shamis, R. A. Lerner, C. F. Barbas III, and D. Shabat, *Angew. Chem. Int. Ed.* **2005**, *44*, 716–720). In a 25 mL round-bottom flask, 4-hydroxybenzyl alcohol (0.3 g, 2.3 mmol) and DIPEA (400 µL, 0.3 g, 2.3 mmol, 1 eq)

were dissolved in dry THF (3 mL). The flask was put in an ice bath, 4-nitrophenyl chloroformate (0.52 g, 2.5 mmol, 1.05 eq) in dry THF (2 mL) was added dropwise and the mixture was stirred at room temperature for 1 hr. After filtration of the formed white precipitate (DIPEA·HCl salt), the solvent was removed *in vacuo* and the residue was redissolved in EtOAc (50 mL). The organic layer was washed with water and brine (both 20 mL), dried with $MgSO_4$, filtrated and the solvent was removed *in vacuo*. Purification was achieved using column chromatography (flash silica, 10% THF in chloroform, the compound was added to the top of the column in 30 mL eluent) and precipitation (acetone → pentane). This yielded pure **62** (0.57 g, 2.0 mmol, 84%) as a white solid. Its spectral characteristics match the reported data.

In a 10 mL round-bottom flask, **62** (50 mg, 0.17 mmol) was dissolved in dry THF (1 mL) under an Ar atmosphere. After the addition of phosgene (179 µL of a 1.9 M solution in toluene, 0.34 mmol, 2 eq) the flask was sealed and the mixture was stirred at room temperature for 15 hrs. The solvent was removed *in vacuo* and the resulting oil was flushed with toluene (3×) and chloroform. This yielded the analogous chloroformate as a colorless oil which was used immediately without further purification.

^{1}H-NMR ($CDCl_3$): δ = 8.33 (d, 2H, Ar*H*), 7.49 (d, 2H, Ar*H*), 7.48 (d, 2H, Ar*H*), 7.33 (d, 2H, Ar*H*), 5.31 (s, 2H, CH_2) ppm.

Subsequently, in a 25 mL round-bottom flask, doxorubicin hydrochloride (86 mg, 0.15 mmol) was dissolved in dry THF (2 mL) and a solution of the chloroformate (61 mg, 0.17 mmol, 1.2 eq) in dry THF (4 mL) was added. After the addition of DIPEA (115 µL, 0.65 mmol, 4.4 eq) the mixture was stirred at room temperature for 23 hrs. The solution was filtered over Celite and the solvent was removed *in vacuo*. The residue was redissolved in chloroform (60 mL) and washed with water (2×) and brine (all 20 mL). The organic layer was dried with $MgSO_4$, filtrated and the solvent was removed *in vacuo*. Purification was achieved using column chromatography (flash silica) using a gradient of 2% MeOH in chloroform to 12% MeOH in chloroform. This yielded pure **63** (70 mg, 82 µmol, 56%) as an orange solid.

^{1}H-NMR ($CDCl_3$): δ = 13.97 (s, 1H, ArO*H*), 13.22, (s, 1H, ArO*H*), 8.30 (d, 2H, Ar*H*), 8.03 (d, 1H, Ar*H*), 7.78 (t, 1H, Ar*H*), 7.46 (d, 2H, Ar*H*), 7.39 (m, 3H, Ar*H*), 7.23 (d, 2H, Ar*H*), 5.50 (d, 1H, OC*H*O), 5.28 (s, 1H, CCHHC*H*), 5.17 (d, 1H, N*H*), 5.04 (s, 2H, ArCH_2O), 4.75 (s, 2H, CH_2OH), 4.54 (s, 1H, CO*H*), 4.14 (m, 1H, C*H*CH_3), 4.08 (s, 3H, OCH_3), 3.87 (m, 1H, NHC*H*), 3.66 (s, 1H, C*H*OH), 3.27 (d, 1H, ArC*H*H), 3.02 (s, 1H, CH_2O*H*), 3.00 (d, 1H, ArCH*H*), 2.33 (d, 1H, CC*H*H), 2.17 (d, 1H, CCH*H*), 1.98 (br, 1H,

CHO*H*), 1.88 (m, 1H, NHCHC*H*H), 1.77 (m, 1H, NHCHCH*H*), 1.29 (d, 3H, CHCH_3) ppm. The assignments were confirmed by 2D (^{1}H-^{1}H) correlation spectroscopy (gCOSY). ^{13}C-NMR ($CDCl_3$): δ = 213.8, 187.1, 186.6, 161.0, 156.2, 155.6, 155.3, 155.2, 150.9, 150.3, 145.6, 135.8, 135.4, 135.1, 133.6, 133.5, 129.5, 125.4, 125.3, 121.7, 120.8, 119.9, 118.5, 111.6, 111.4, 100.7, 69.7, 69.6, 67.2, 65.8, 65.5, 56.7, 47.0, 35.6, 34.0, 30.2, 16.8 ppm. ESI-MS: *m/z* Calc. 858.21; Obs. $[M+Na]^+$ 881.42.

In a 25 mL round-bottom flask, *cis*-5,6-diamino-*trans*-cyclooctene* **35** (16.5 mg, 71 µmol, 1.1 eq) was dissolved in dry THF (4 mL) under an Ar atmosphere. A solution of **63** (55 mg, 64 µmol) in dry THF (4 mL) was added, the flask was sealed and the mixture was stirred at room temperature for 1 hr. The solvent was removed *in vacuo* and the residue was purified using RP-HPLC (CH_3CN / H_2O with 0.1% formic acid) while monitoring at λ = 253 and 317 nm. The gradient comprised, % CH_3CN (min): 28 (1-11), 28 to 100 (11-12), 100 (12-13), 100 to 28 (13-14), 28 (14-15) This yielded pure (E)-cyclooctene-doxorubicin conjugate ***64*** (19 mg, 22 µmol, 35%) as an orange solid after freeze-drying.

^{1}H-NMR ($CDCl_3$ / MeOD-d_4 95:5): δ = 7.98 (d, 1H, Ar*H*), 7.74 (t, 1H, Ar*H*), 7.35 (d, 1H, Ar*H*), 7.23 (d, 2H, Ar*H*), 6.99 (d, 2H, Ar*H*), 5.75 (m, 1H, C*H*=CH), 5.64 (m, 1H, CH=C*H*), 5.43 (s, 1H, OC*H*O), 5.23 (s, 1H, CCHHC*H*), 4.97 (d, 1H, ArC*H*HO), 4.91 (d, 1H, ArCH*H*O), 4.71 (s, 2H, CH_2OH), 4.09 (m, 1H, C*H*CH_3), 4.08 (s, 3H, OCH_3), 3.80 (m, 1H, NHC*H*), 3.70 (m, 1H, NHC*H*CHNH_2), 3.56 (s, 1H, C*H*OH), 3.40 (m, 1H, C*H*NH_2), 3.22 (d, 1H, ArC*H*H), 2.98 (d, 1H, ArCH*H*), 2.38-1.96 (m, 10H, *trans*-cyclooctene CH_2, CC*HH*), 1.77 (m, 2H, NHCHC*HH*), 1.22 (d, 3H, CHCH_3) ppm. ^{13}C-NMR (MeOD-d_4): δ = 213.2, 186.1, 185.9, 160.8, 156.5, 155.7, 155.1, 154.5, 150.6, 135.7, 134.6, 134.2, 134.1, 133.6, 133.1, 132.8, 128.6, 121.2, 119.7, 119.0, 118.7, 110.8, 110.6, 100.8, 78.0, 76.0, 69.7, 68.7, 67.2, 65.4, 64.3, 57.3, 55.9, 55.6, 35.9, 35.7, 35.5, 32.6, 31.6, 29.4, 27.6, 15.9 ppm. ESI-MS: *m/z* Calc. 859.32; Obs. $[M+H]^+$ 860.50. FT-IR (ATR): υ = 3347, 2972, 2940, 2872, 1676, 1617, 1579, 1525, 1503, 1444, 1429, 1413, 1346, 1285, 1261, 1201, 1134, 1069, 1016, 985, 952, 916, 894, 875, 836, 821, 799, 765, 737, 721, 706, 693, 673 cm^{-1}.

* estimated batch purity 60%

*Synthesis of (*E*)-cyclooctene-doxorubicin conjugate* ***68***

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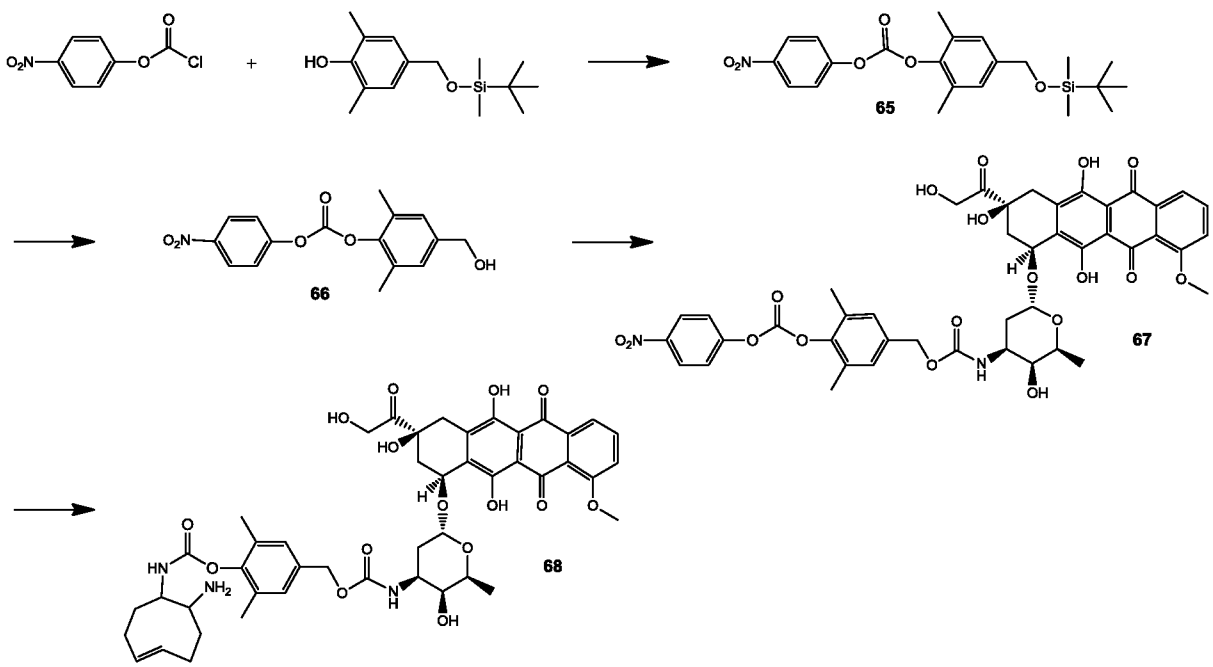

In a 25 mL round-bottom flask, 4-(*t*-butyldimethylsilyloxymethyl)-2,6-dimethylphenol (84 mg, 0.32 mmol; Y. H. Choe, C. D. Conover, D. Wu, M. Royzen, Y. Gervacio, V. Borowski, M. Mehlig, R. B. Greenwald, *J. Controlled Release* **2002**, *79*, 55–70) and DIPEA (111 µL, 82 mg, 0.63 mmol, 2 eq) were dissolved in dry THF (0.5 mL) and the mixture was cooled on an ice bath. 4-Nitrophenyl chloroformate (132 mg, 0.63 mmol, 2 eq) in dry THF (0.5 mL) was added dropwise and the mixture was stirred at 45 °C for 2 hrs. Since ^{1}H-NMR indicated $\eta \approx 78\%$, additional DIPEA (55 µL, 41 mg, 0.31 mmol, 1 eq) and 4-nitrophenyl chloroformate (66 mg, 0.31 mmol, 1 eq) were added and the mixture was stirred at 45 °C for 30 min. After filtration of the formed white precipitate (DIPEA·HCl salt) over Celite, the solvent was removed *in vacuo* and the residue was redissolved in EtOAc (140 mL). The organic layer was washed with water and brine (both 45 mL), dried with $MgSO_4$, filtrated and the solvent was removed *in vacuo*. Purification was achieved using column chromatography (flash silica) using a gradient of 1:1 chloroform / pentane to chloroform. This yielded pure 2,6-dimethyl-4-(*t*-butyldimethylsilyloxymethyl)phenyl 4-nitrophenyl carbonate (**65**) (75 mg, 0.17 mmol, 55%) as a colorless solid.

^{1}H-NMR ($CDCl_3$): δ = 8.31 (d, 2H, Ar*H*), 7.48 (d, 2H, Ar*H*), 7.06 (s, 2H, Ar*H*), 4.68 (s, 2H, CH_2), 2.28 (s, 6H, $ArCH_3$), 0.95 (s, 9H, CCH_3), 0.11 (s, 6H, $SiCH_3$) ppm. ^{13}C-NMR ($CDCl_3$): δ = 155.5, 150.4, 146.9, 145.6, 139.8, 129.5, 126.6, 125.4, 121.6, 64.3, 26.0, 18.4, 16.2, -5.3 ppm. ESI-MS: *m/z* Calc. 431.18; Obs. $[M+2H\text{-}TBDMS\text{-}H_2O]^+$ 300.17. FT-IR (ATR): υ = 2954, 2929, 2885, 2857, 1777, 1616, 1594, 1526, 1491, 1471, 1462, 1444,

1407, 1346, 1323, 1292, 1220, 1177, 1164, 1128, 1100, 1003, 947, 910, 883, 834, 815, 775, 736, 700, 673, 657 cm^{-1}.

In a 25 mL round-bottom flask, **65** (166 mg, 0.38 mmol) was dissolved in ethanol (6 mL) and the solution was cooled on an ice bath. Conc. HCl in ethanol (1% v/v, 4.5 mL) was added and the mixture was stirred at room temperature for 75 min. The solvent was removed *in vacuo* and the residue was flushed with chloroform. This yielded pure 2,6-dimethyl-4-(hydroxymethyl)phenyl 4-nitrophenyl carbonate (**66**) (136 mg, max. 0.38 mmol, 100%) as a colorless oil.

^{1}H-NMR ($CDCl_3$): δ = 8.32 (d, 2H, Ar*H*), 7.47 (d, 2H, Ar*H*), 7.13 (s, 2H, Ar*H*), 4.65 (s, 2H, CH_2), 2.30 (s, 6H, ArCH_3) ppm. ^{13}C-NMR ($CDCl_3$): δ = 155.4, 150.4, 147.3, 145.6, 139.3, 130.0, 127.5, 125.4, 121.6, 64.6, 16.1 ppm. ESI-MS: *m/z* Calc. 317.09; Obs. $[M+H-H_2O]^+$ 300.17. FT-IR (ATR): υ = 3555, 3366, 3119, 3086, 2924, 2867, 1772, 1616, 1593, 1523, 1490, 1454, 1380, 1346, 1324, 1311, 1293, 1220, 1176, 1164, 1126, 1056, 1035, 1003, 955, 941, 910, 884, 857, 845, 764, 732, 702, 679, 664 cm^{-1}.

In a 10 mL round-bottom flask, **66** (51 mg, 0.16 mmol) was dissolved in dry THF (1 mL) under an Ar atmosphere. After the addition of phosgene (180 µL of a 1.9 M solution in toluene, 0.34 mmol, 2 eq) the flask was sealed and the mixture was stirred at room temperature for 15 hrs. The solvent was removed *in vacuo* and the resulting oil was flushed with toluene (3×) and chloroform. This yielded the analogous chloroformate as a colorless oil which was used immediately without further purification.

^{1}H-NMR ($CDCl_3$): δ = 8.33 (d, 2H, Ar*H*), 7.48 (d, 2H, Ar*H*), 7.16 (s, 2H, Ar*H*), 5.24 (s, 2H, CH_2), 2.31 (s, 6H, CH_3) ppm.

Subsequently, in a 10 mL round-bottom flask, doxorubicin hydrochloride (89 mg, 0.15 mmol) was dissolved in dry THF (2 mL) and a solution of the chloroformate (max. 0.16 mmol, 1.1 eq) in dry THF (4 mL) was added. After the addition of DIPEA (118 µL, 88 mg, 0.67 mmol, 4.4 eq) the mixture was stirred at room temperature for 24 hrs. The solution was filtered over Celite and the solvent was removed *in vacuo*. The residue was redissolved in chloroform (120 mL) and washed with water (2×) and brine (all 40 mL). The organic layer was dried with $MgSO_4$, filtered and the solvent was removed *in vacuo*. Purification was achieved using column chromatography (flash silica) using a gradient of 2% MeOH in chloroform to 3% MeOH in chloroform. This yielded pure **67** (82 mg, 92 µmol, 61%) as an orange solid.

^{1}H-NMR ($CDCl_3$): δ = 13.98 (s, 1H, ArO*H*), 13.25, (s, 1H, ArO*H*), 8.30 (d, 2H, Ar*H*), 8.05 (d, 1H, Ar*H*), 7.79 (t, 1H, Ar*H*), 7.45 (d, 2H, Ar*H*), 7.40 (d, 1H, Ar*H*), 7.06

(s, 2H, Ar*H*), 5.51 (s, 1H, OC*H*O), 5.30 (s, 1H, CCHHC*H*), 5.11 (d, 1H, N*H*), 4.97 (s, 2H, ArCH_2O), 4.75 (s, 2H, CH_2OH), 4.53 (s, 1H, CO*H*), 4.14 (m, 1H, C*H*CH_3), 4.08 (s, 3H, OCH_3), 3.87 (m, 1H, NHC*H*), 3.67 (d, 1H, C*H*OH), 3.28 (d, 1H, ArC*H*H), 3.03 (d, 1H, ArCH*H*), 2.98 (t, 1H, CH_2O*H*), 2.33 (d, 1H, CC*H*H), 2.25 (s, 6H, ArCH_3), 2.17 (d, 1H, CCH*H*), 1.87 (m, 2H, NHCHC*H*H, CHO*H*), 1.77 (m, 1H, NHCHCH*H*), 1.29 (d, 3H, CHCH_3) ppm. ^{13}C-NMR ($CDCl_3$): δ = 213.8, 186.9, 186.5, 161.0, 156.1, 155.5, 155.4, 155.3, 150.2, 147.7, 145.6, 135.8, 135.4, 134.8, 133.6, 133.5, 130.1, 128.8, 125.4, 121.6, 120.7, 119.8, 118.5, 111.5, 111.3, 100.7, 77.2, 76.6, 69.7, 69.5, 67.3, 66.0, 65.5, 56.6, 47.0, 35.6, 33.9, 30.1, 16.8, 16.0 ppm. ESI-MS: *m/z* Calc. 886.24; Obs. $[M+Na]^+$ 909.33. FT-IR (ATR): υ = 3492, 3431, 3058, 2937, 1777, 1719, 1616, 1579, 1525, 1491, 1444, 1429, 1412, 1381, 1347, 1325, 1283, 1225, 1209, 1183, 1132, 1071, 1015, 982, 948, 917, 879, 858, 847, 820, 791, 765, 734, 702, 681 cm^{-1}.

In a 25 mL round-bottom flask, *cis*-5,6-diamino-*trans*-cyclooctene* (**35**, 14.6 mg, 104 µmol, 1.1 eq) was dissolved in dry THF (4 mL) under an Ar atmosphere. A solution of **67** (82 mg, 92 µmol) in dry THF (2 mL) was added, the flask was sealed and the mixture was stirred at room temperature for 90 min. Since ^{1}H-NMR indicated η ≈ 75%, additional 5,6-diamino-*trans*-cyclooctene (4.4 mg, 31 µmol, 0.3 eq) was added and the mixture was stirred at room temperature for 1 hr (eventually η ≈ 90% based on ^{1}H-NMR). The solvent was removed *in vacuo* and part (22 mg) of the residue (100 mg) was purified using RP-HPLC (CH_3CN / H_2O with 0.1% formic acid) while monitoring at λ = 253 and 317 nm. The gradient comprised, % CH_3CN (min): 25 to 45 (1-11), 45 to 100 (11-12), 100 (12-13), 100 to 25 (13-14), 25 (14-15) This yielded pure *cis*-(E)-cyclooctene-doxorubicin conjugate **68** (10 mg, 11 µmol, 55%) as an orange solid after freeze-drying.

^{1}H-NMR ($CDCl_3$ / MeOD-d_4 95:5): δ = 8.02 (d, 1H, Ar*H*), 7.80 (t, 1H, Ar*H*), 7.41 (d, 1H, Ar*H*), 6.98 (s, 2H, Ar*H*), 5.82 (m, 1H, C*H*=CH), 5.66 (m, 1H, CH=C*H*), 5.49 (s, 1H, OC*H*O), 5.32 (s, 1H, CCHHC*H*), 4.95 (d, 1H, ArC*H*HO), 4.88 (d, 1H, ArCH*H*O), 4.77 (s, 2H, CH_2OH), 4.14 (m, 1H, C*H*CH_3), 4.08 (s, 3H, OCH_3), 3.84 (m, 1H, NHC*H*), 3.74 (m, 1H, NHC*H*CHNH_2), 3.60 (s, 1H, C*H*OH), 3.40 (m, 1H, C*H*NH_2), 3.26 (d, 1H, ArC*H*H), 3.02 (d, 1H, ArCH*H*), 2.38-1.96 (m, 10H, *trans*-cyclooctene CH_2, CC*HH*), 2.11 (s, 6H, ArCH_3), 1.81 (m, 2H, NHCHC*HH*), 1.28 (d, 3H, CHCH_3) ppm. ^{13}C-NMR ($CDCl_3$ / MeOD-d_4 9:1): δ = 213.9, 187.1, 186.7, 161.0, 155.8, 155.3, 153.9, 147.7, 135.8, 135.4, 133.8, 133.6, 133.2, 132.8, 130.9, 128.3, 120.8, 119.7, 118.5, 111.5, 111.3, 100.6, 76.4, 69.3, 69.0, 67.9, 67.4, 66.2, 65.2, 57.8, 56.6, 55.9, 46.8, 36.5, 36.1, 35.7, 33.7, 32.6, 29.9, 28.0, 25.5, 16.6, 15.9 ppm. ESI-MS: *m/z* Calc. 887.35; Obs. $[M+H]^+$ 888.58. FT-IR (ATR): υ = 3432, 2940, 1678,

1617, 1582, 1515, 1412, 1350, 1285, 1236, 1201, 1140, 1075, 1017, 983, 949, 912, 873, 836, 820, 799, 765, 729, 672 cm^{-1}.

* estimated batch purity 98%

Example 3

Stability and reactivity of tetrazine Activators

Hydrolytic stability tests of tetrazines

10 μL of a solution of the specific tetrazine in DMSO (25 mM) was diluted with PBS buffer (3 mL) (or a mixture of PBS and acetonitrile in case the aqueous solubility was too low). This solution was filtered and, the decrease of the absorption band at 525 nm was monitored using UV spectroscopy. The rate of hydrolysis and half-life time was determined from these data.

Reactivity of tetrazines towards trans-cyclooct-4-ene-1-ol (axial isomer)

A competition experiment was performed to determine the reactivity ratio of a specific tetrazine and 3-(5-acetamido-2-pyridyl)-6-(2-pyridyl)-1,2,4,5-tetrazine (that was chosen as the reference tetrazine), in the inverse-electron demand Diels-Alder reaction with *trans*-cyclooct-4-ene-1-ol ("minor" isomer with OH in axial position, see: Whitham *et al. J. Chem. Soc. (C)*, 1971, 883-896)).

To acetonitrile (0.100 mL) was added 5 μL of a solution of the specific tetrazine in DMSO (25 mM) and 5 μL of a solution of the reference tetrazine in DMSO (25 mM). This mixture was diluted with water (0.9 mL), and the absolute amounts of both tetrazines were determined by HPLC-MS/PDA analysis. Subsequently, a solution of *trans*-cyclooct-4-ene-1-ol (axial isomer) in DMSO (25 μL 2.5 mM) was slowly added, and the mixture was stirred for 5 min. Again, the absolute amounts of both tetrazines were determined by HPLC-MS/PDA analysis, and conversions for both tetrazines was calculated. From these conversions, the reactivity ratio ($R=k_{2,TCO}/k_{2,Ref}$) of both tetrazines was calculated using the mathematical procedure from Ingold and Shaw (*J. Chem. Soc.*, **1927**, 2918-2926).

The table below demonstrates how the reactivity and stability profile of tetrazines can be tailored to certain specifications by varying the substituents.

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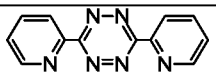
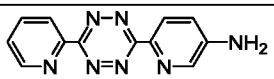
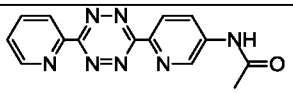
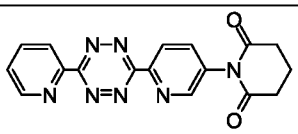
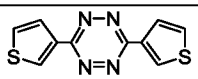
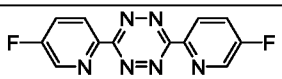
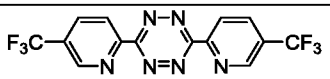
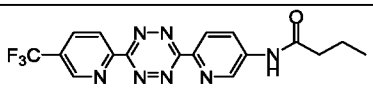
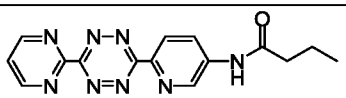
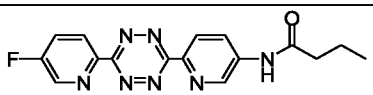
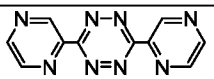
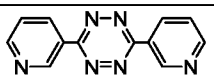
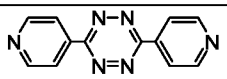
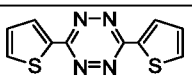
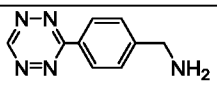
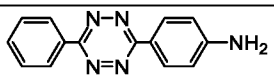
tetrazine	stability in PBS at 20°C $t_{1/2}$ (hrs)	Reactivity ratio ($R=k_{2,TZ}/k_{2,Ref}$)
 **2**	44	1.17
	340	0.4
 **5**	80	1
	24	1.6
	>300*	<0.01*
	115	1.07
	3.6*	5.3*
	35*	1.84*
	3.2	2.7
	117	0.95
	0.68	1.5
	>150	0.19
	2.4	0.83
	>300*	<0.01*
	183	0.77
	>300*	<0.01*

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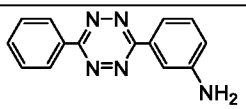
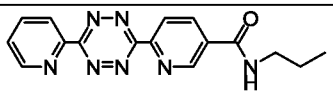
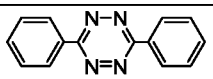
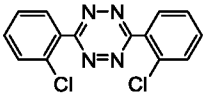
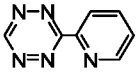
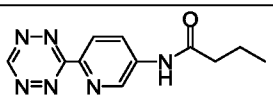
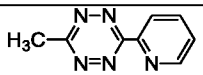
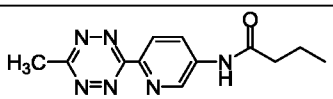
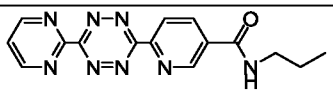
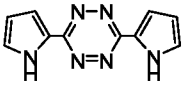
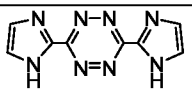
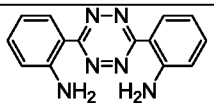
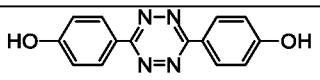
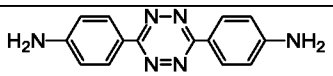
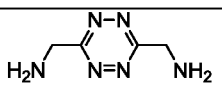
	>300*	<0.01*
	4	1.76
	>300*	<0.01*
	>300*	<0.01*
	2.7	3.06
	10.3	2.8
 7	230	0.25
	300	0.18
	0.42	2
	>300*	<0.01*
	n.d.	1.2
 9	>300*	<0.01*
 11	>300*	<0.01*
 13	>300*	<0.01*
 20	16	n.d.

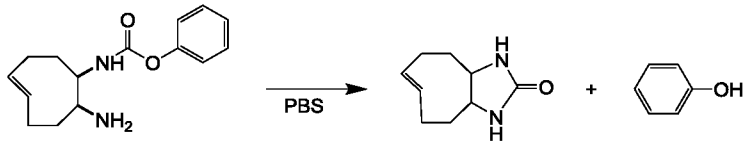
* This value was determined in a 50:50 mixture of PBS and acetonitrile.

Example 4

Stability and reactivity of *trans*-cyclooctene model prodrugs and prodrugs

Stability

10 µL of a solution of the specific *trans*-cyclooctene derivative in dioxane (25 mM) was diluted with PBS buffer (3 mL), and this solution was stored at 20°C in the dark. The fate of the TCO compound was monitored by HPLC-MS analysis, and an estimation of the half-life time was made based on the release of the model prodrug.



Reactivity of trans-cyclooctene derivatives towards bis(2-pyridyl)-1,2,4,5-tetrazine: second-order rate constant determination

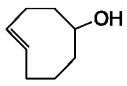
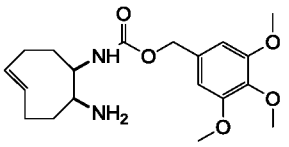
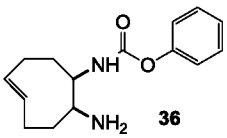
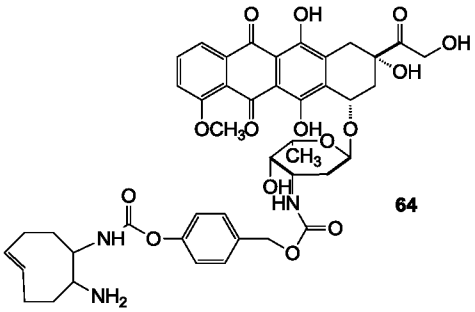
The kinetics of the inverse-electron demand Diels-Alder reaction of a *trans*-cyclooctene derivative with 3-(5-acetamido-2-pyridyl)-6-(2-pyridyl)-1,2,4,5-tetrazine, performed in acetonitrile at 20°C, was determined using UV-visible spectroscopy. A cuvette was filled with acetonitrile (3 mL) and equilibrated at 20°C. 3-(5-Acetamido-2-pyridyl)-6-(2-pyridyl)-1,2,4,5-tetrazine (2.50×10^{-7} mol) was added, followed by the *trans*-cyclooctene derivative (2.50×10^{-7} mol). The decay of the absorption at λ=540 nm was monitored, and from this curve the second-order rate constant, k_2, was determined assuming second order rate kinetics.

Reactivity of trans-cyclooctene derivatives towards bis(2-pyridyl)-1,2,4,5-tetrazine: competition experiment

A competition experiment was performed to determine the reactivity ratio of a specific *trans*-cyclooctene derivative and *trans*-cyclooct-4-ene-1-ol (axial isomer) (that was chosen as the reference), in the inverse-electron demand Diels-Alder reaction with bis(2-pyridyl)-1,2,4,5-tetrazine.

To acetonitrile (0.05 mL) was added a solution of the specific *trans*-cyclooctene derivative in dioxane (5 µL 25 mM; 1.25×10^{-7} mol) and a solution of the reference *trans*-cyclooctene in dioxane (5 µL 25 mM; 1.25×10^{-7} mol). This mixture was diluted with water (0.45 mL). Subsequently, a solution of bis(2-pyridyl)-1,2,4,5-tetrazine

(6.25×10^{-8} mol) in a mixture of acetonitrile (0.05 mL) and water (0.45 mL) was slowly added while stirring vigorously. After addition, the mixture was stirred for an additional 5 min. The conversion of both *trans*-cyclooctene derivatives was determined by HPLC-MS/PDA analysis, and from these conversions, the reactivity ratio ($R=k_{2,TCO}/k_{2,Ref}$) of the specific *trans*-cyclooctene derivative was calculated using the mathematical procedure from Ingold and Shaw (*J. Chem. Soc.*, **1927**, 2918-2926).

***trans*-cyclooctene derivative**	**stability in PBS at 20°C, $t_{1/2}$**	**rate contant* k_2 (M^{-1} s^{-1})**	**reactivity ratio** ($R=k_{2,TCO}/k_{2,Ref}$)**
 axial isomer	> 3 days	577	1
 **37**	> 10 days	300	
 **36**	27 hrs	240	
 **64**	24 hrs@20°C 3.6 hrs@37°C		

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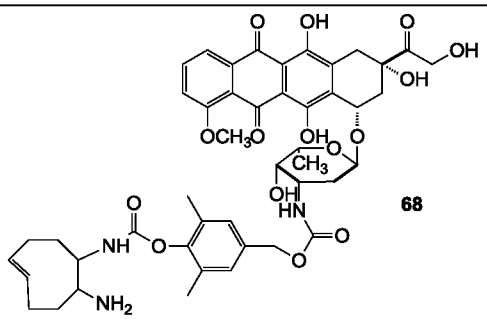
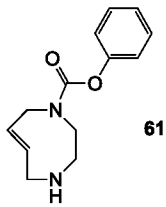
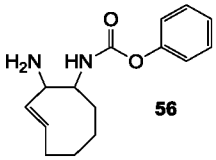
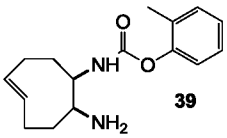
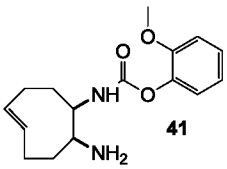
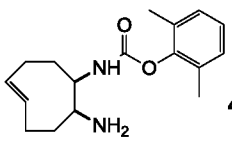
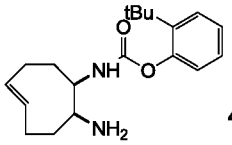
 68	26 days@20°C 6.2 days@37°C		
 61	>> 20 days	47	
 56	5 days	44.4	
 39	8 days		
 41	21 hrs		
 43	>> 20 days		
 45	>> 20 days		

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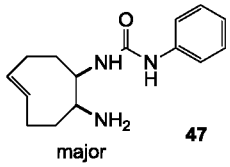
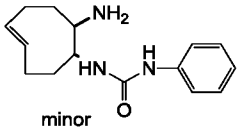
 major **47**	>> 20 days	106	
 minor **47**	>> 20 days	56	

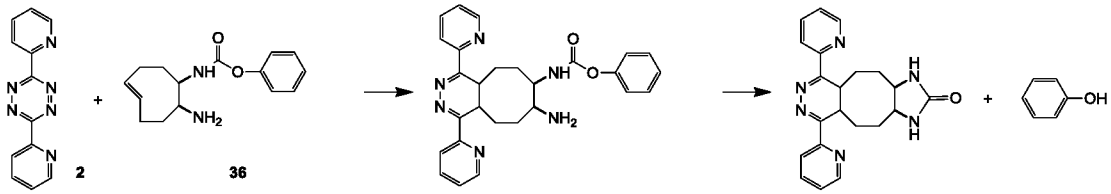
* determined by UV-visible spectroscopy in acetonitrile at 20°C

** determined by a competition experiment

Example 5

Activation of model prodrugs

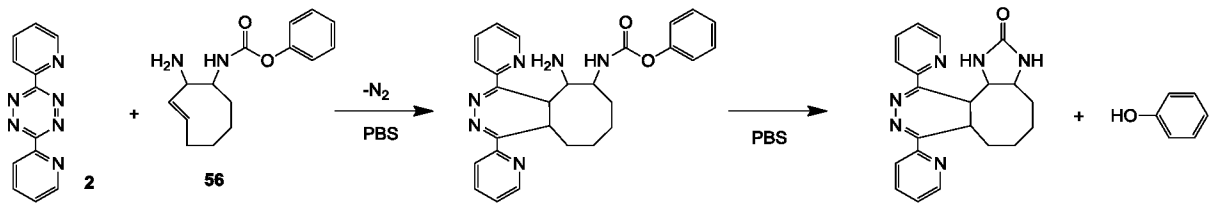
*3,6-Bis(2-pyridinyl)-1,2,4,5-tetrazine (**2**) and phenyl ((E)-8-aminocyclooct-4-en-1-yl)carbamate (**36**)*



2 **36**

3,6-Bis(2-pyridinyl)-1,2,4,5-tetrazine (**2**, 2.50×10^{-7} mol) was dissolved in PBS buffer (1 mL). Next, phenyl ((*E*)-8-aminocyclooct-4-en-1-yl)carbamate (**36**, 2.50×10^{-7} mol) was added. The solution was stirred at 20°C, and the reaction progress was monitored by HPLC-MS analysis, demonstrating nearly instantaneous formation of the rDA-adduct, followed by the formation of the cyclic urea with m/z = +375 Da ($M+H^+$), and release of phenol: λ_{max}=270 nm. The half-life time of this release was 40 min.

*3,6-Bis(2-pyridinyl)-1,2,4,5-tetrazine (**2**) and phenyl ((E)-2-aminocyclooct-3-en-1-yl)carbamate (**56**)*

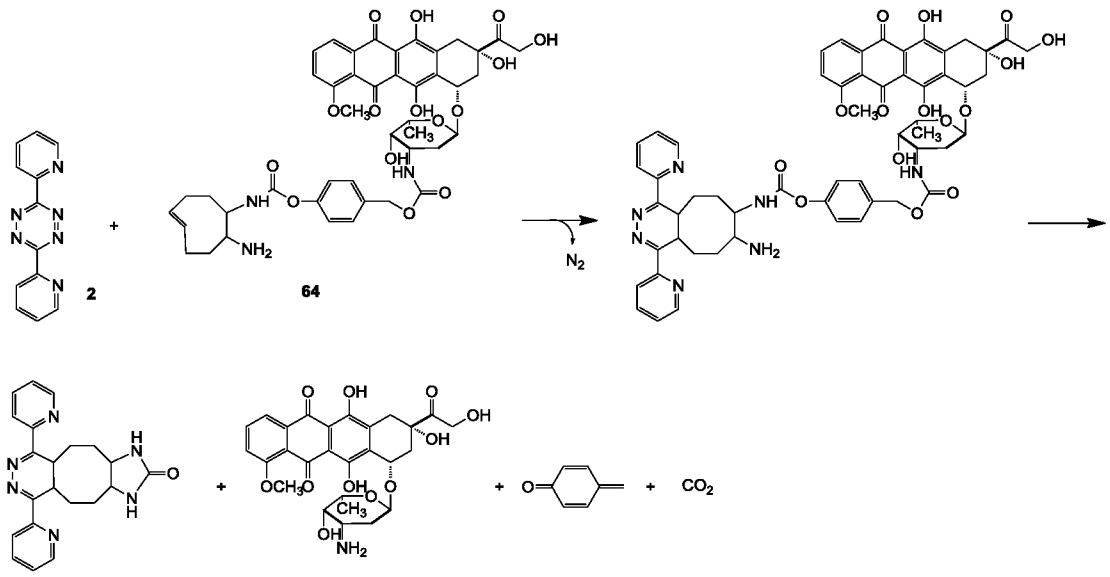


3,6-Bis(2-pyridinyl)-1,2,4,5-tetrazine (**2**, 2.50×10^{-7} mol) was dissolved in PBS buffer (1 mL). Next, phenyl ((*E*)-2-aminocyclooct-3-en-1-yl)carbamate (**56**, 2.50×10^{-7} mol) was added. The solution was stirred at 20°C, and the reaction progress was monitored by HPLC-MS analysis, proving almost instantaneous formation of the rDA-adduct, followed by the formation of the cyclic urea with m/z = +375 Da ($M+H^+$), and release of phenol: λ_{max}=270 nm. The half-life time of this release was 40 min.

Example 6

Activation of doxorubicin prodrugs

*3,6-Bis(2-pyridinyl)-1,2,4,5-tetrazine (**2**) and (*E*)-cyclooctene-doxorubicin conjugate (**64**)*

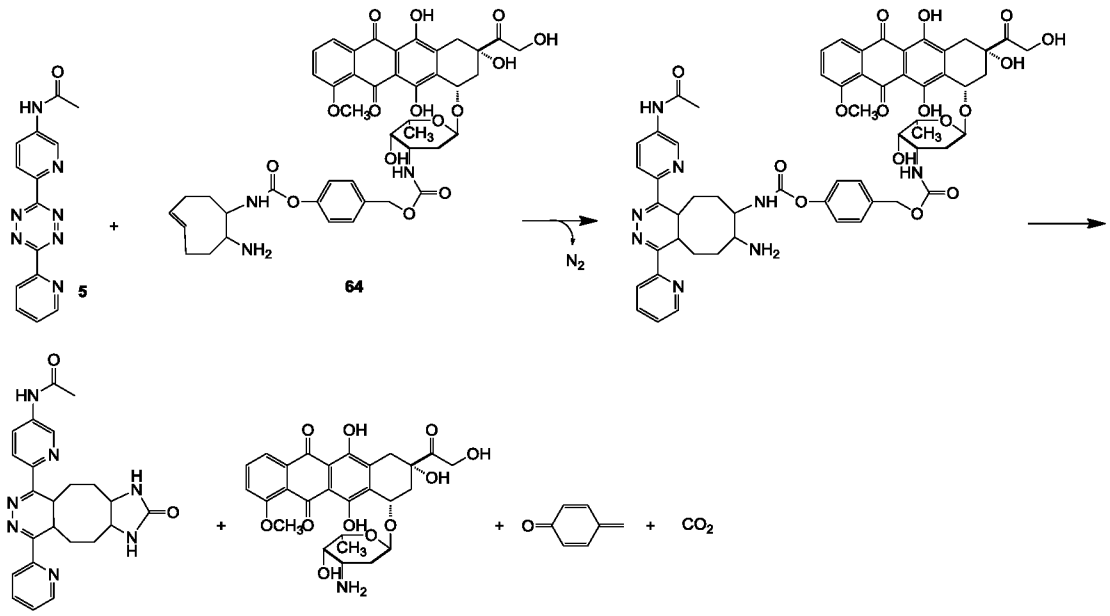


3,6-Bis(2-pyridinyl)-1,2,4,5-tetrazine (**2**, 1.18×10^{-5} g; 5.00×10^{-8} mol) was dissolved in PBS buffer (1 mL). Next, *cis*-(E)-cyclooctene-doxorubicin conjugate (**64**, 2.67×10^{-5} g; 2.50×10^{-8} mol) was added. The solution was stirred at 20°C, and the reaction progress was monitored by HPLC-MS analysis, proving the formation of the cyclic urea with

m/z = +375 Da ($M+H^+$), and release of doxorubicin: m/z = +544 Da ($M+H^+$) and λ_{max}=478 nm. The half-life time of this release was 2 hrs.

Performing this reaction at 37°C yielded a doxorubicin release half-life time of 40 min.

*3-(5-Acetamido-2-pyridyl)-6-(2-pyridyl)-1,2,4,5-tetrazine (**5**) and (*E*)-cyclooctene-doxorubicin conjugate (**64**)*



Same procedure as previous reaction.

After 1 hr at 20°C, 30% doxorubicin was released.

*3-(5-Butyramido-2-pyridyl)-6-(2-pyrimidyl)-1,2,4,5-tetrazine and (*E*)-cyclooctene-doxorubicin conjugate (**64**)*

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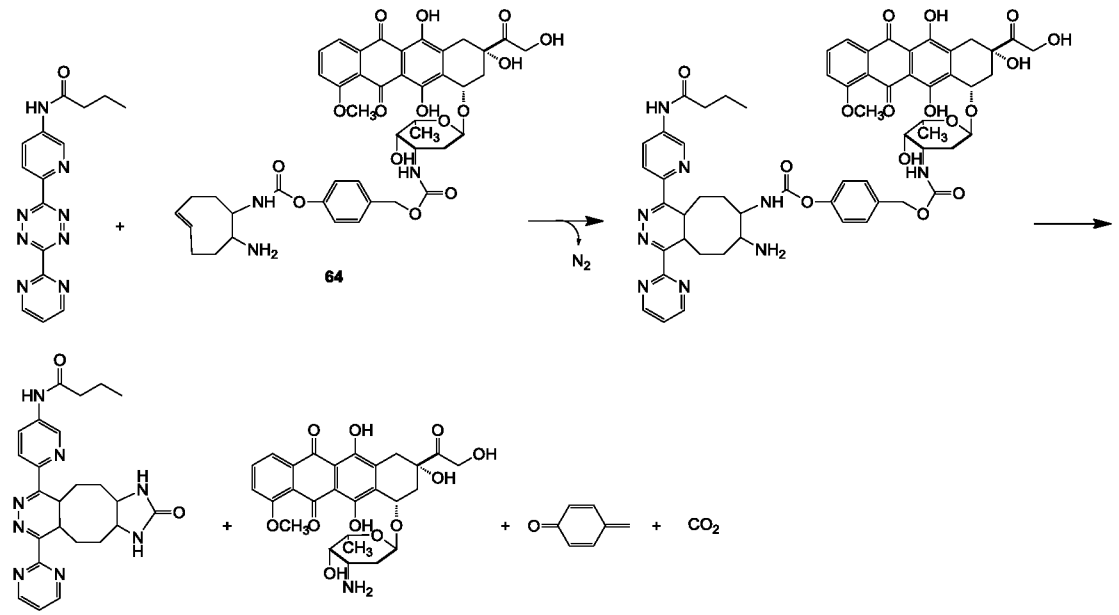
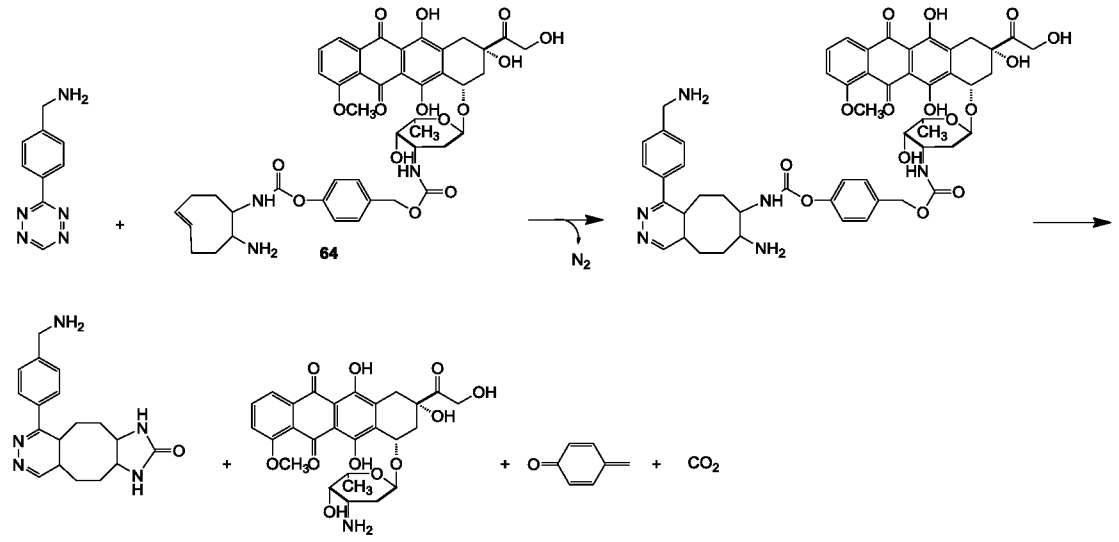


Same procedure as previous reaction.

After 1 hr at 20°C, 20% doxorubicin was released.

*4-(1,2,4,5-Tetrazin-3-yl)phenylmethanamine and (*E*)-cyclooctene-doxorubicin conjugate (**64**)*



Same procedure as previous reaction.

After 1 hr at 20°C, 50% doxorubicin was released.

*3-Methyl-6-(2-pyridyl)-1,2,4,5-tetrazine (7) and (*E*)-cyclooctene-doxorubicin conjugate (**64**)*

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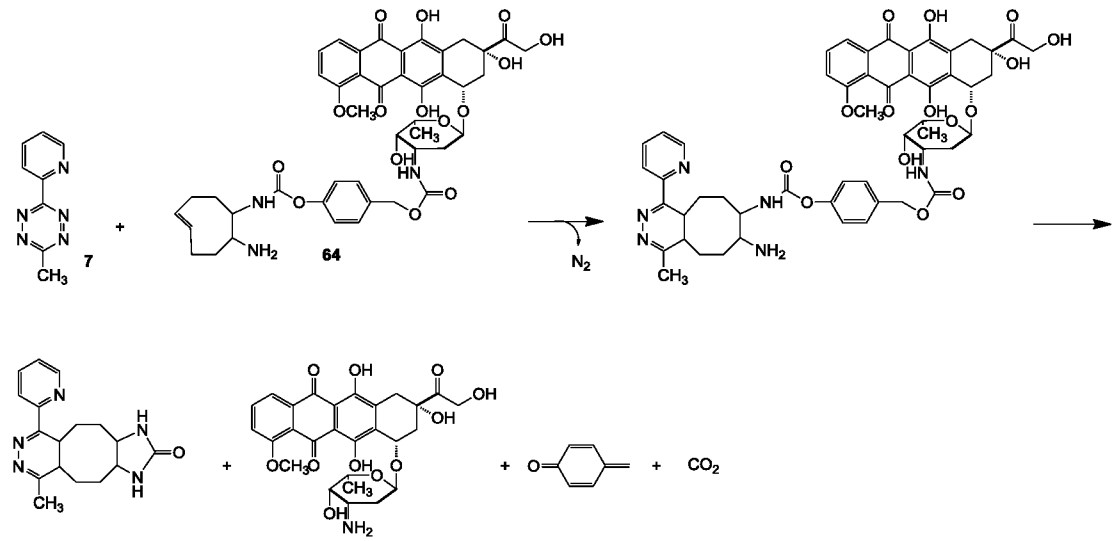
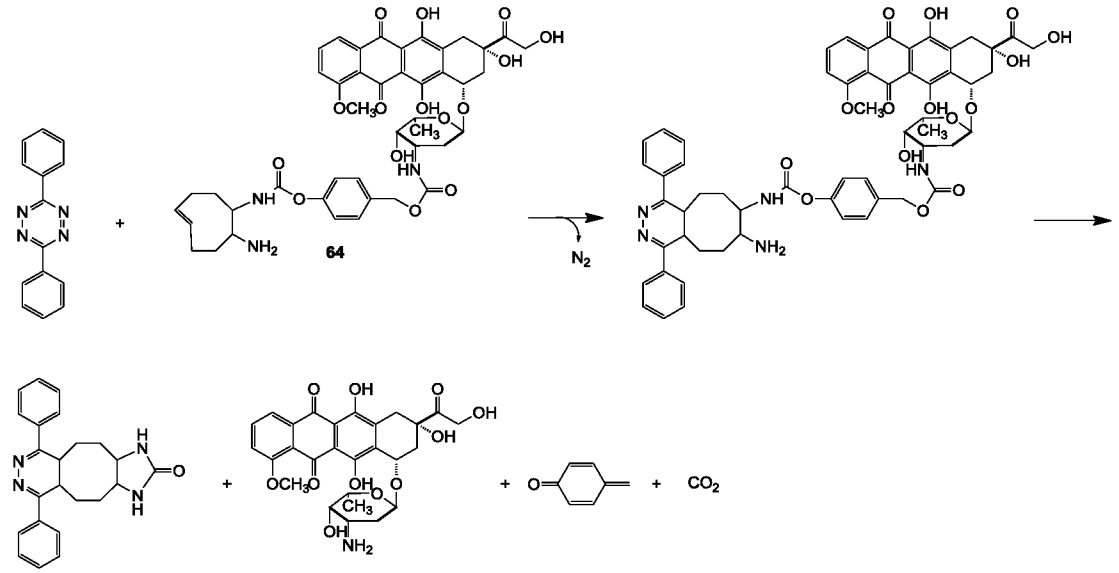


Same procedure as previous reaction.

After 1 hr at 20°C, 52% doxorubicin was released.

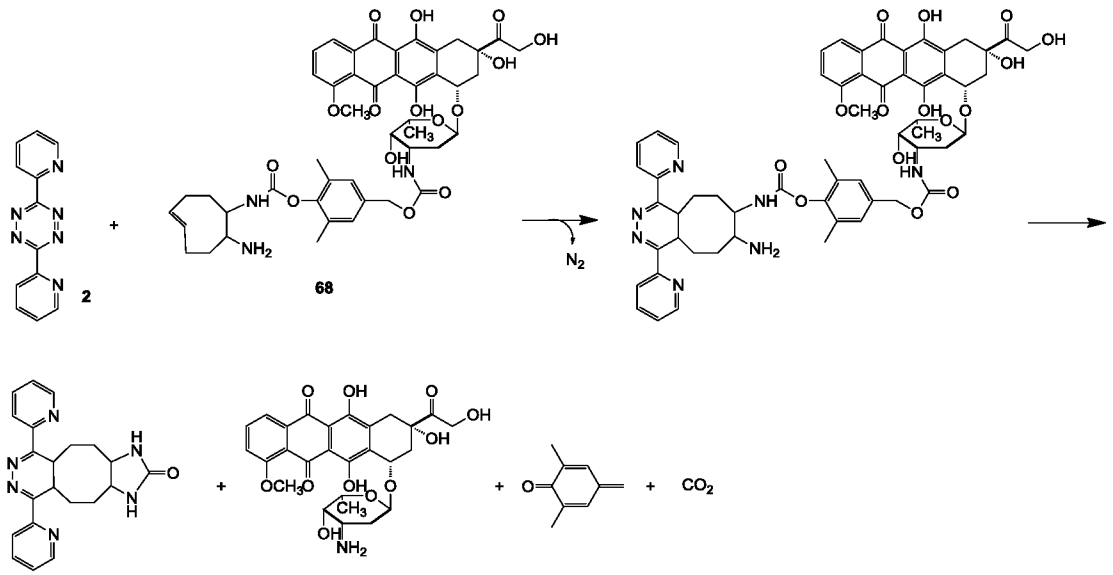
*3,6-Diphenyl-1,2,4,5-tetrazine and (*E*)-cyclooctene-doxorubicin conjugate (**64**)*



Same procedure as previous reaction.

After 1 hr at 20°C, 48% doxorubicin was released.

*3,6-Bis(2-pyridinyl)-1,2,4,5-tetrazine (**2**) and (E)-cyclooctene-doxorubicin conjugate (**68**)*



3,6-Bis(2-pyridinyl)-1,2,4,5-tetrazine (**2**, 1.18×10^{-5} g; 5.00×10^{-8} mol) was dissolved in PBS buffer (1 mL). Next, (E)-cyclooctene-doxorubicin conjugate (**68**, 2.67×10^{-5} g; 2.50×10^{-8} mol) was added. The solution was stirred at 20°C, and the reaction progress was monitored by HPLC-MS analysis, proving the formation of the cyclic urea with m/z = +375 Da ($M+H^+$), and release of doxorubicin: m/z = +544 Da ($M+H^+$) and λ_{max}=478 nm. The half-life time of this release was 4 days.

Performing this reaction at 37°C yielded a half-life time of 16 hrs.

Example 7

Cell proliferation assay with doxorubicin prodrug 64 and tetrazine 29

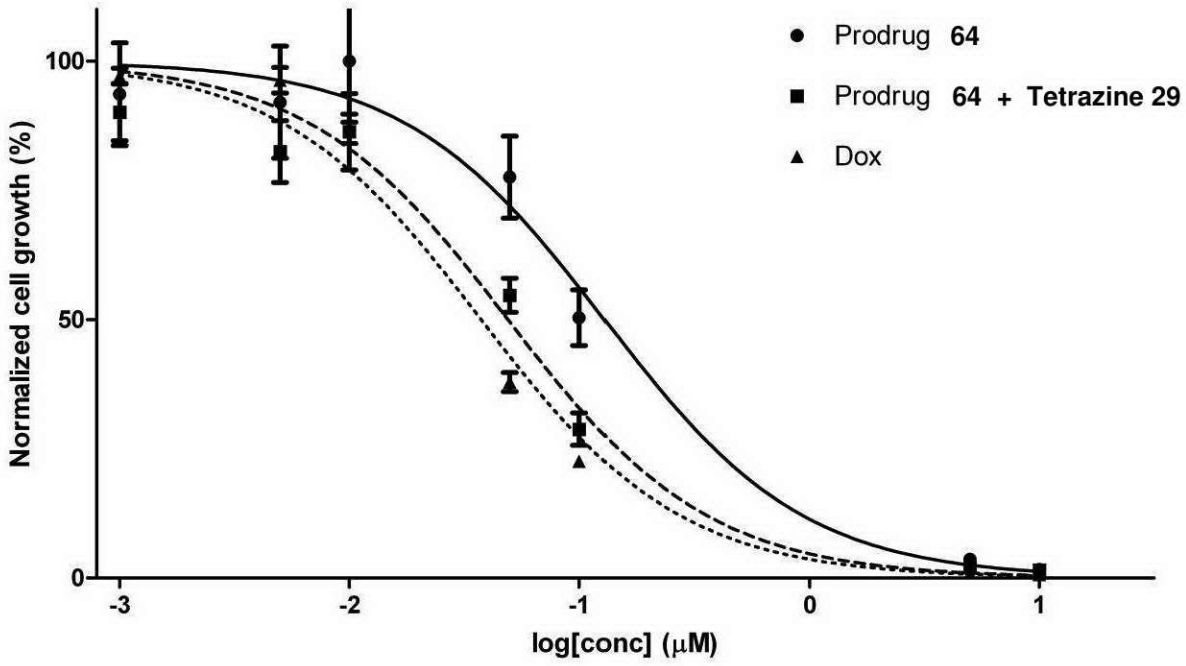
A431 squamous carcinoma cells were maintained in a humidified CO_2 (5%) incubator at 37 °C in DMEM (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum and 0.05% glutamax (Invitrogen) in the presence of penicillin and streptomycin. The cells were plated in 96-well plates (Nunc) at a 2000 cells/well density 24 hrs prior to the experiment. Doxorubicin (Dox) and the prodrug **64** (1 mM in DMSO) were serially diluted in pre-warmed culture medium immediately before the experiment and added to the wells (200 µL final volume; t = 0). The prodrug was either added alone or in combination with 10 µM tetrazine **29**. After 6 hrs incubation at 37 °C the medium was gently aspirated, 200 µL fresh culture medium was added to each well and the cells were incubated for 66 hrs more. In a parallel experiment, a solution of tetrazine **29** (2 mM in PBS) was serially diluted (from 1 mM to 1 nM) in pre-warmed culture medium and added to A431 cells

in a 96-well plate, which was incubated at at 37 °C for 72 hrs. At the end of each experiment, the cell proliferation was assessed by an MTT assay. Briefly, methylthiazolyldiphenyltetrazolium bromide (MTT) was dissolved in PBS at 5 mg/ml, filtered through 0.22 µm and 25 µl was added to each well. After 120 min incubation at 37 °C, the medium was gently aspirated. The formed formazan crystals were dissolved in 100 µl DMSO and the absorbance was measured with a plate reader (BMG Labtech) at 560 nm. IC_{50} values (± standard error; see table) were derived from the normalized cell growth curves (see figure) generated with GraphPad Prism (version 5.01). The cell proliferation assays shows a significant toxicity increase when A431 cells are exposed to a combination of the prodrug **64** and tetrazine **29** (IC_{50} = 49 ± 4 nM) compared to the prodrug alone (IC_{50} = 128 ± 17 nM) or the tetrazine alone (IC_{50} > 100 µM). This confirms that doxorubicin is released following the retro Diels-Alder reaction between the trans-cyclooctene of the prodrug and the tetrazine Activator.

*IC_{50} values for doxorubicin (Dox) and prodrug **64** with and without activation by tetrazine **29** (10 µM) determined in A431 cell line.*

Compound	**IC_{50} (µM)**
Dox	0.038 ± 0.003[a]
Prodrug **64**	0.128 ± 0.017[a]
Prodrug **64** + tetrazine **29** (10 µM)	0.049 ± 0.004[a]
Tetrazine **29**	> 100[b]

[a] 6 h incubation at 37 °C followed by medium replacement; [b] 72 h incubation at 37 °C

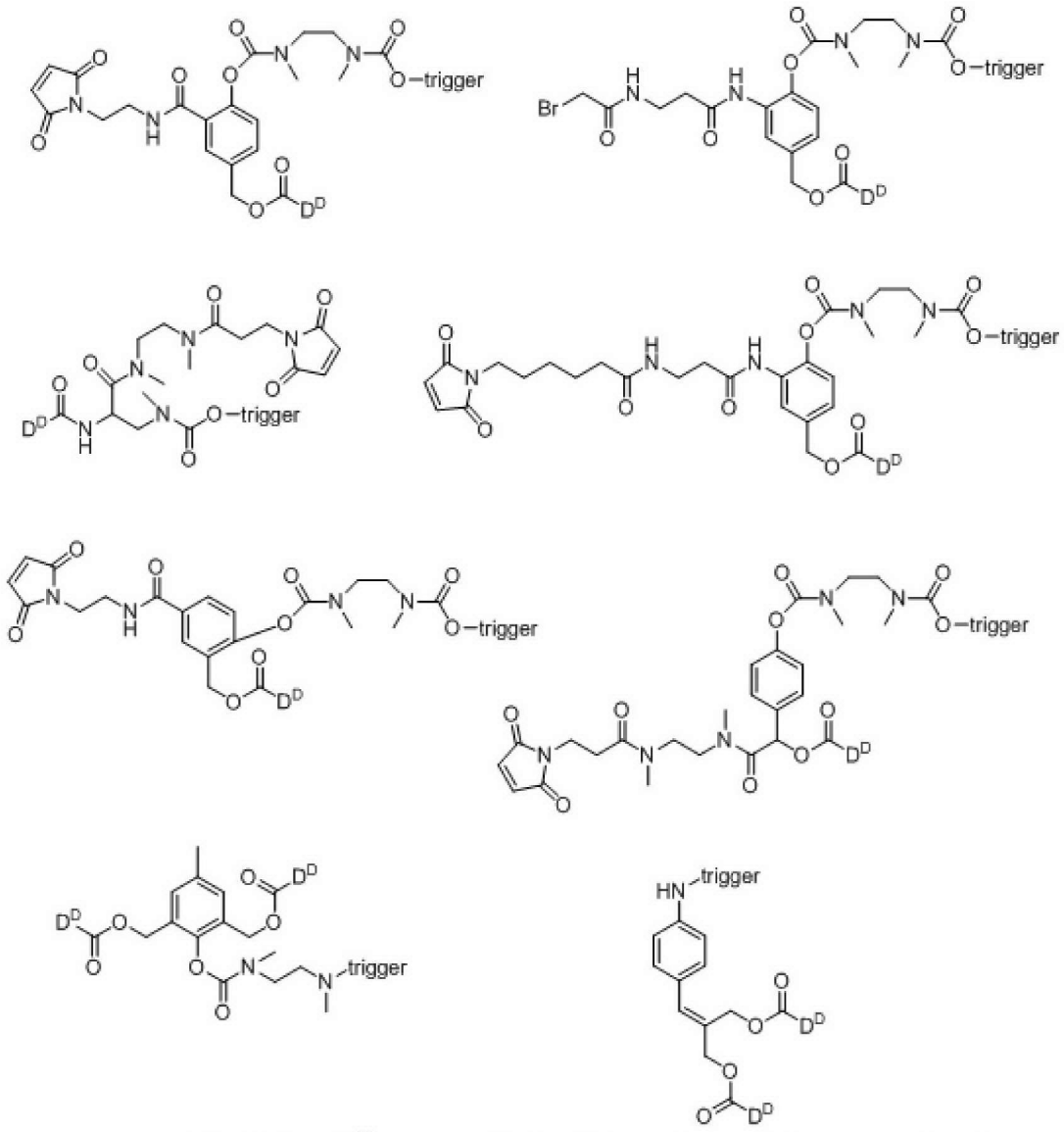


*Cell proliferation assay performed on A431 tumor incubated in the presence of doxorubicin (Dox) and prodrug **64** with and without activation by tetrazine **29** (10 μM).*

5

Example 8.

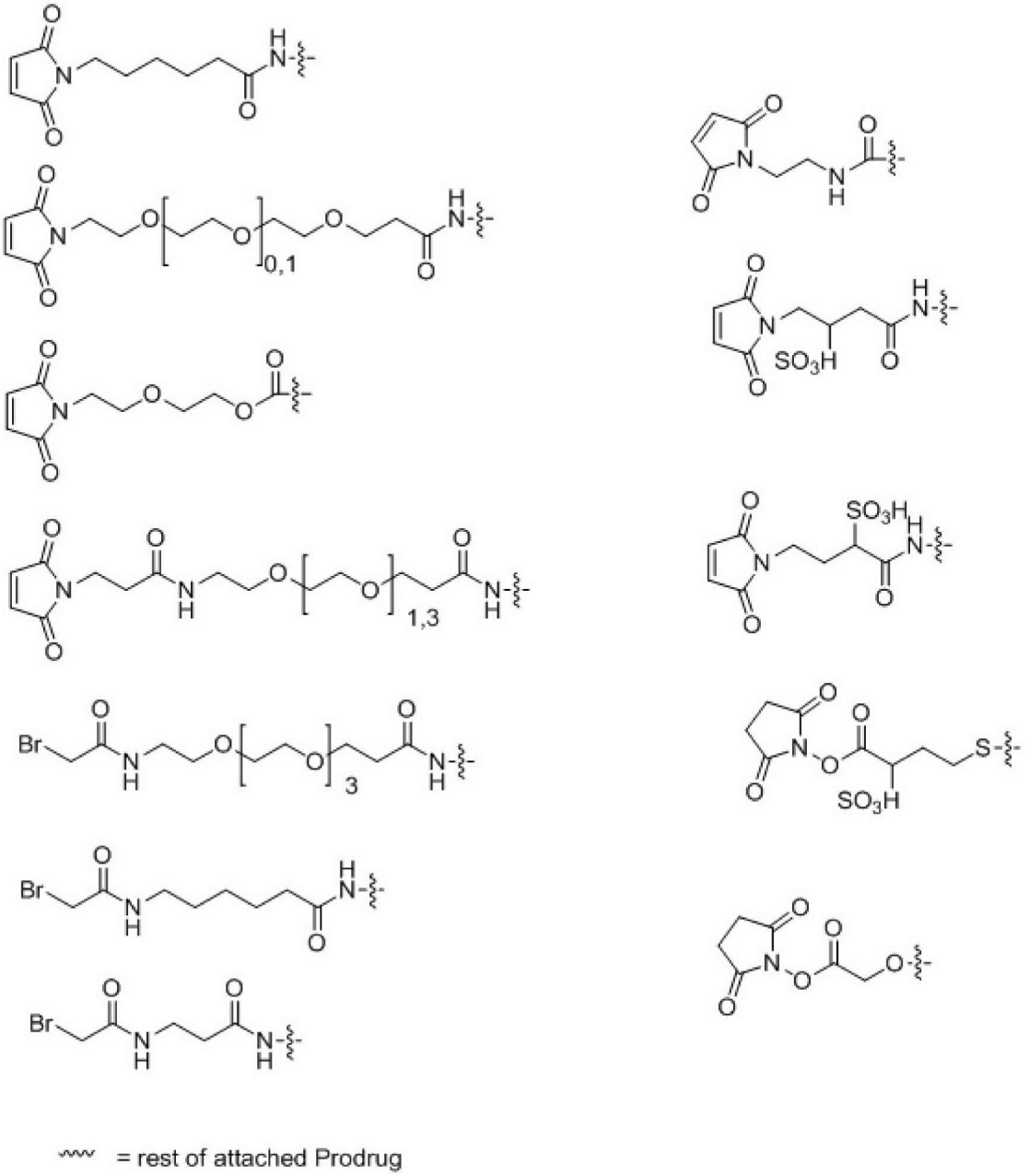
Structures of exemplary L^D moieties



The linkers L^D are so-called self-immolative linkers, meaning that upon reaction of the trigger with the activator the linker will degrade via intramolecular reactions thereby releasing the drug D^D. Some of the above also contain a S^P.

Example 9.

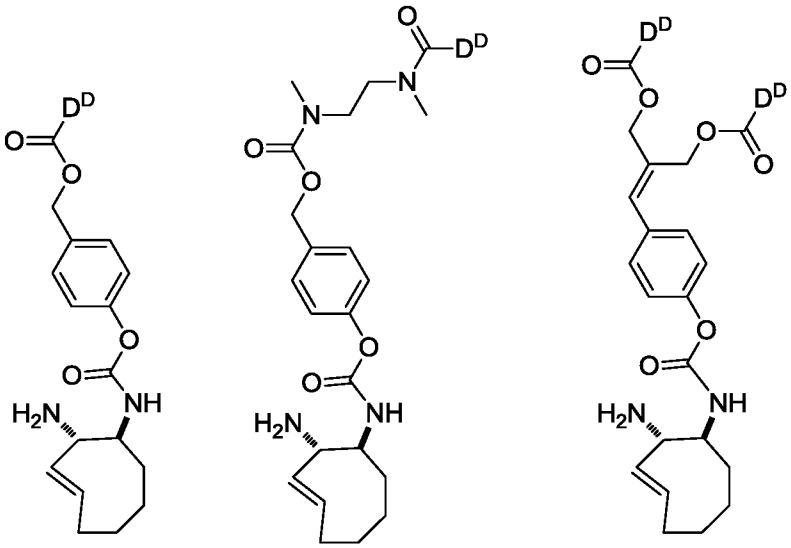
Structures of exemplary S^P moieties



Note that the maleimide, active ester and bromo acetamide groups are active groups to which targeting moieties T^T and masking moieties M^M, optionally via further spacers S^P, can be coupled. Maleimides and bromo acetamide groups typically react with thiols, while active esters are typically suitable for coupling to primary or secondary amines.

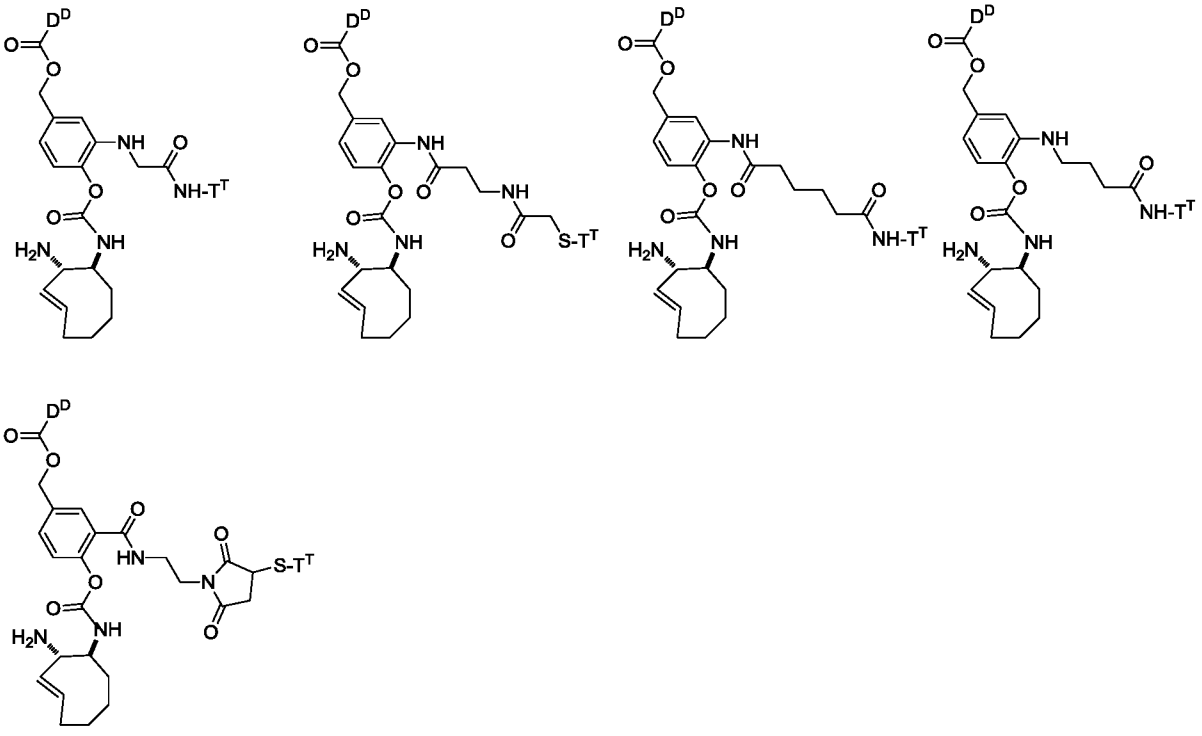
Example 10. Structures of TCO triggers with depicted exemplary L^D moieties.

The T^T featured in this example can optionally be replaced by M^M.



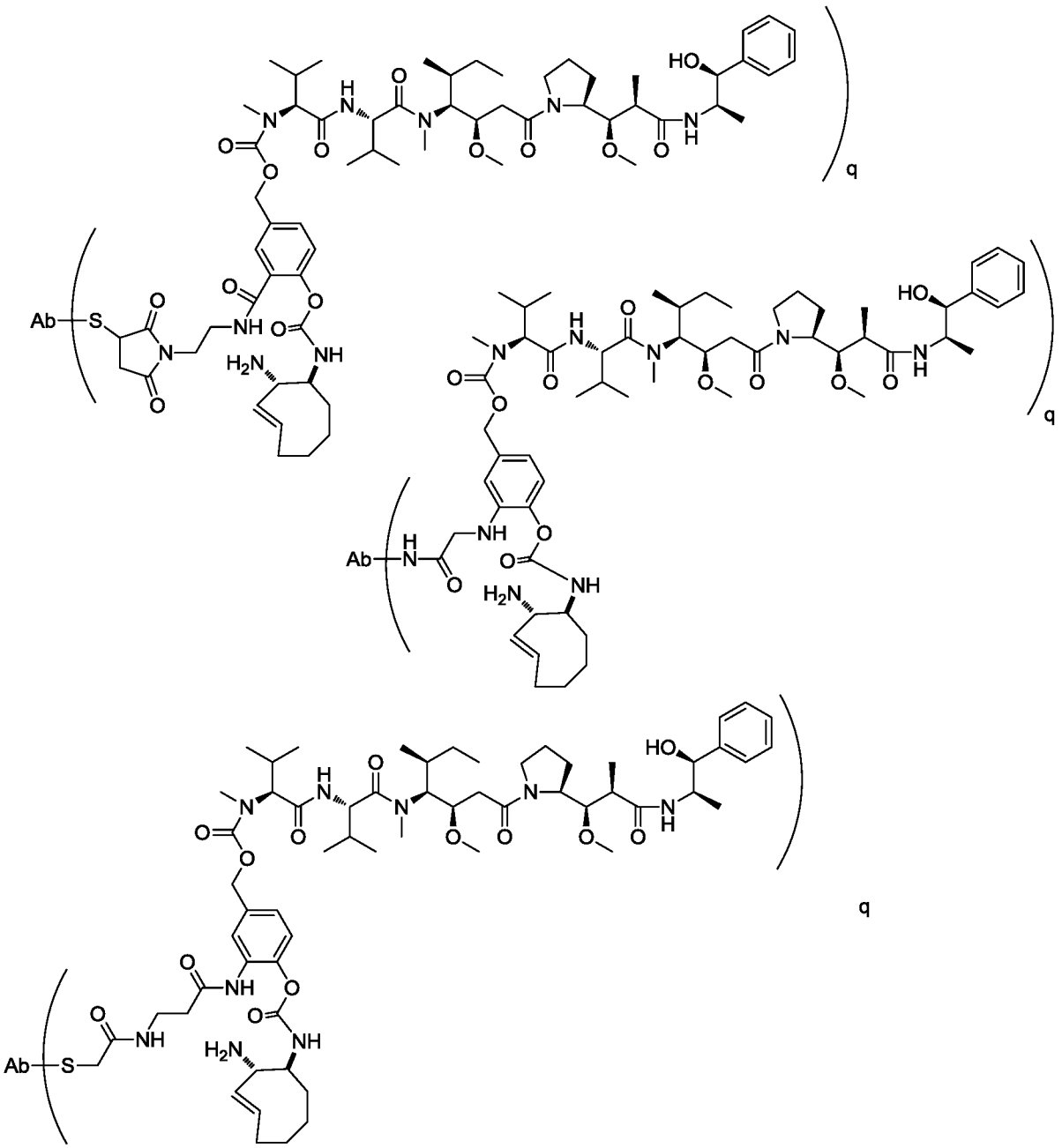
Example 11. Structures of TCO triggers with depicted exemplary L^D and/or S^P moieties

Trigger conjugated to T^T via amine or thiol of T^T. The T^T featured in this example can optionally be replaced by M^M.



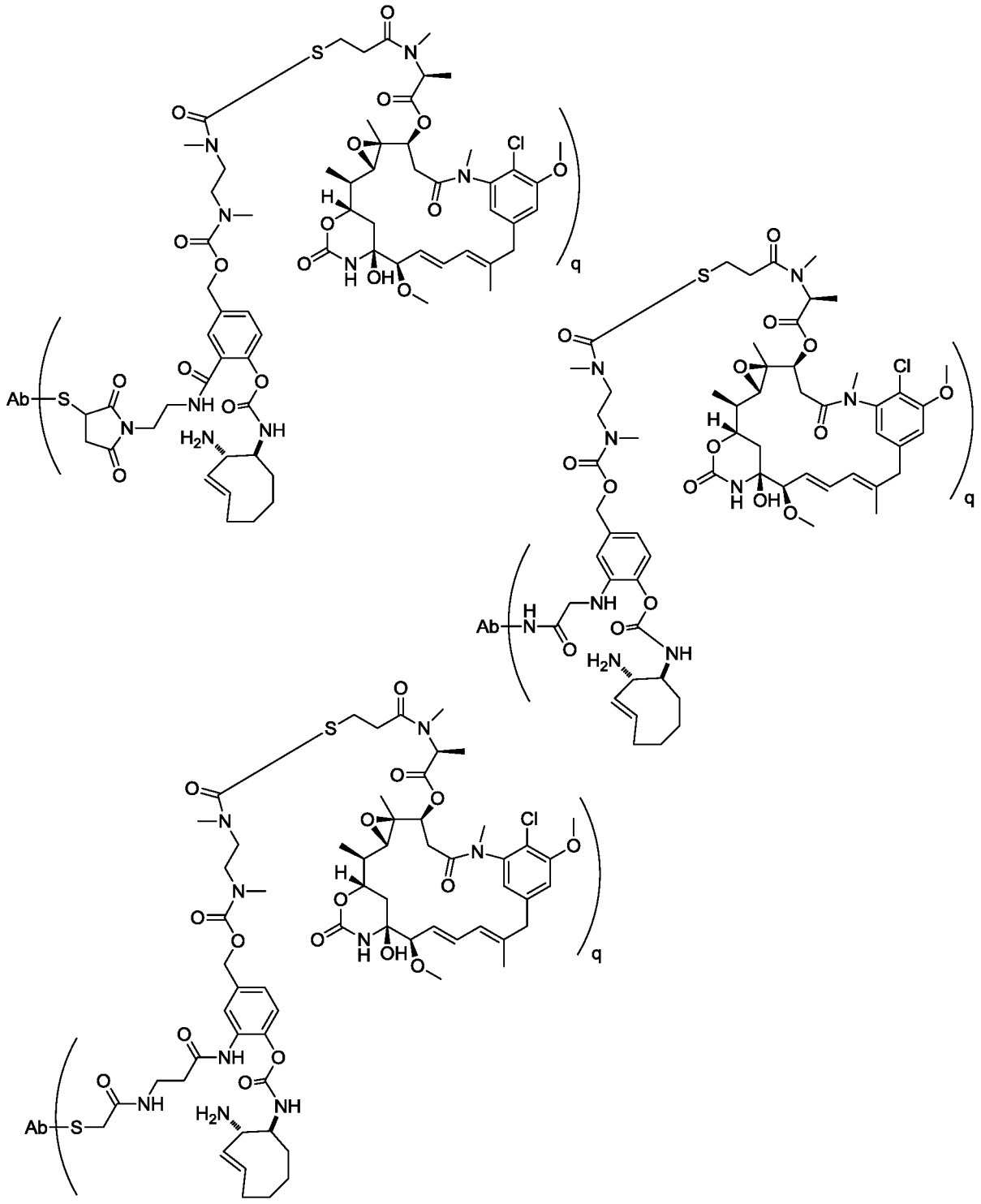
Example 12. Structures of antibody-drug conjugates

Auristatin E (MMAE) toxin is attached via a self immolative linker L^D to a TCO trigger and via S^P to a targeting antibody or fragment (conjugated through cysteine or lysine residue). Ab = antibody or antibody fragment; q = Ab modification # and is typically between 1 and 10.



Example 13. Structures of antibody-drug conjugates.

Maytansine toxin is attached via a self immolative linker L^D to a TCO trigger and via S^P to a targeting antibody or fragment (conjugated through cysteine or lysine residue). Ab = antibody or antibody fragment; q = Ab modification # and is typically between 1 and 10.



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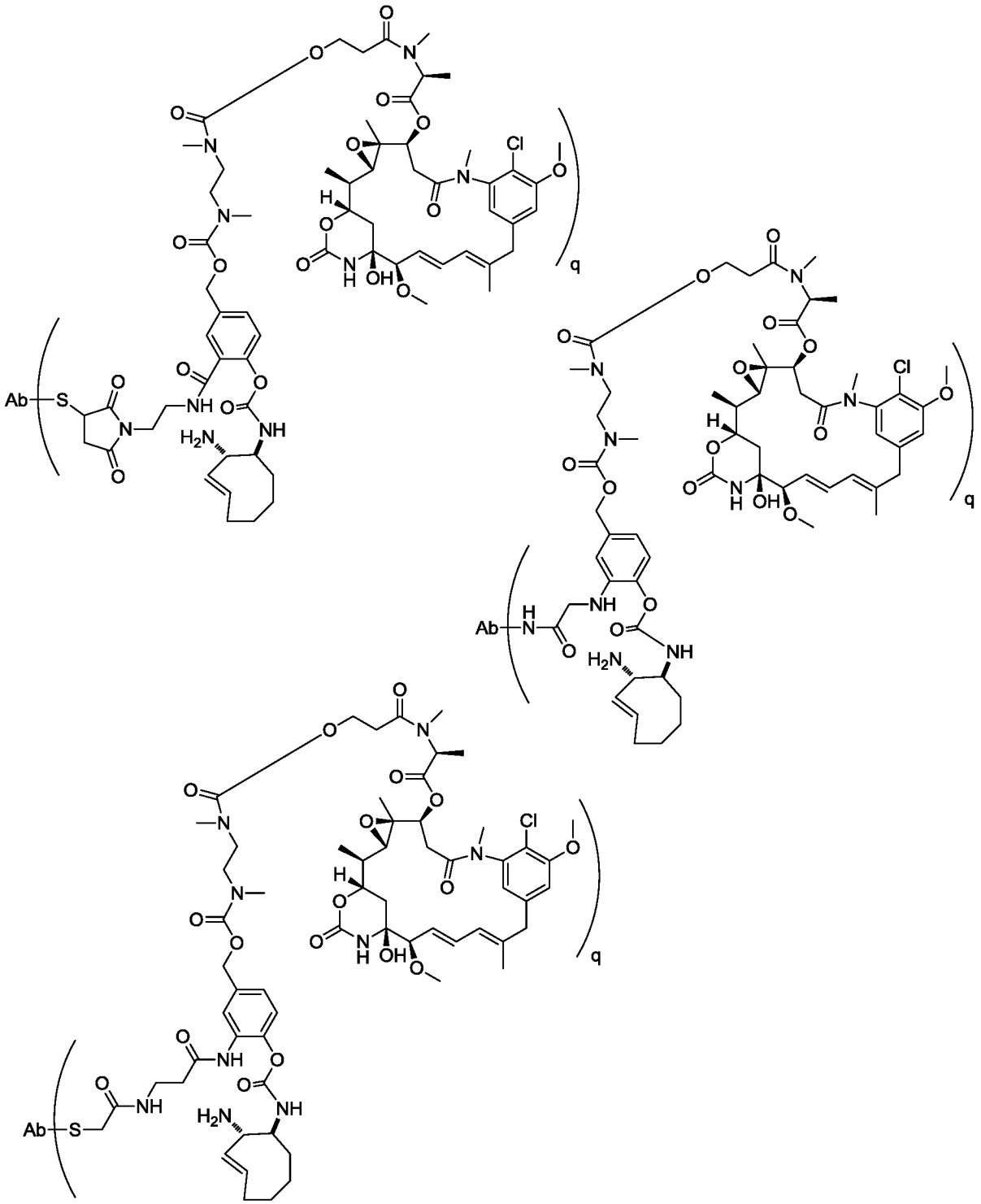
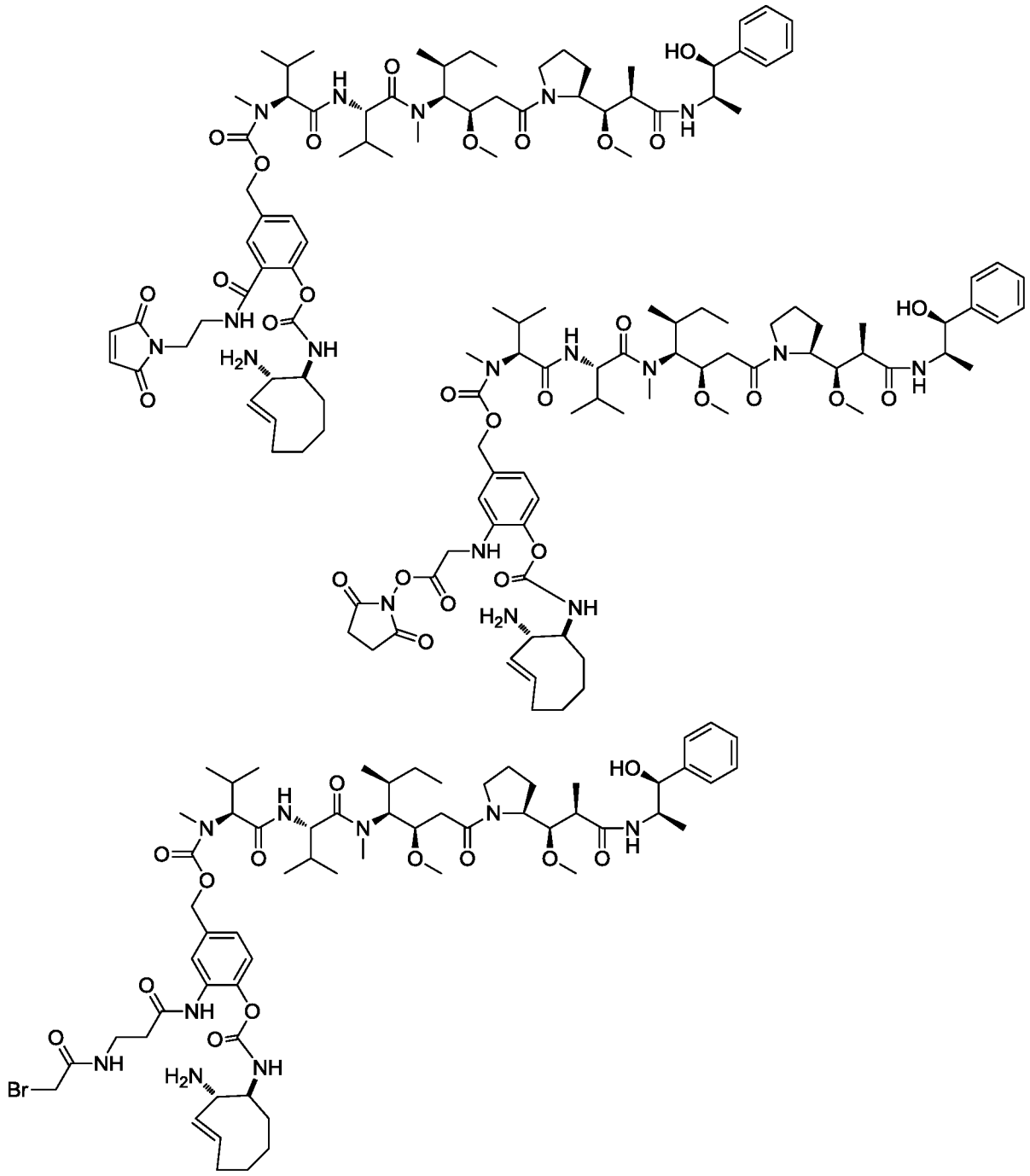


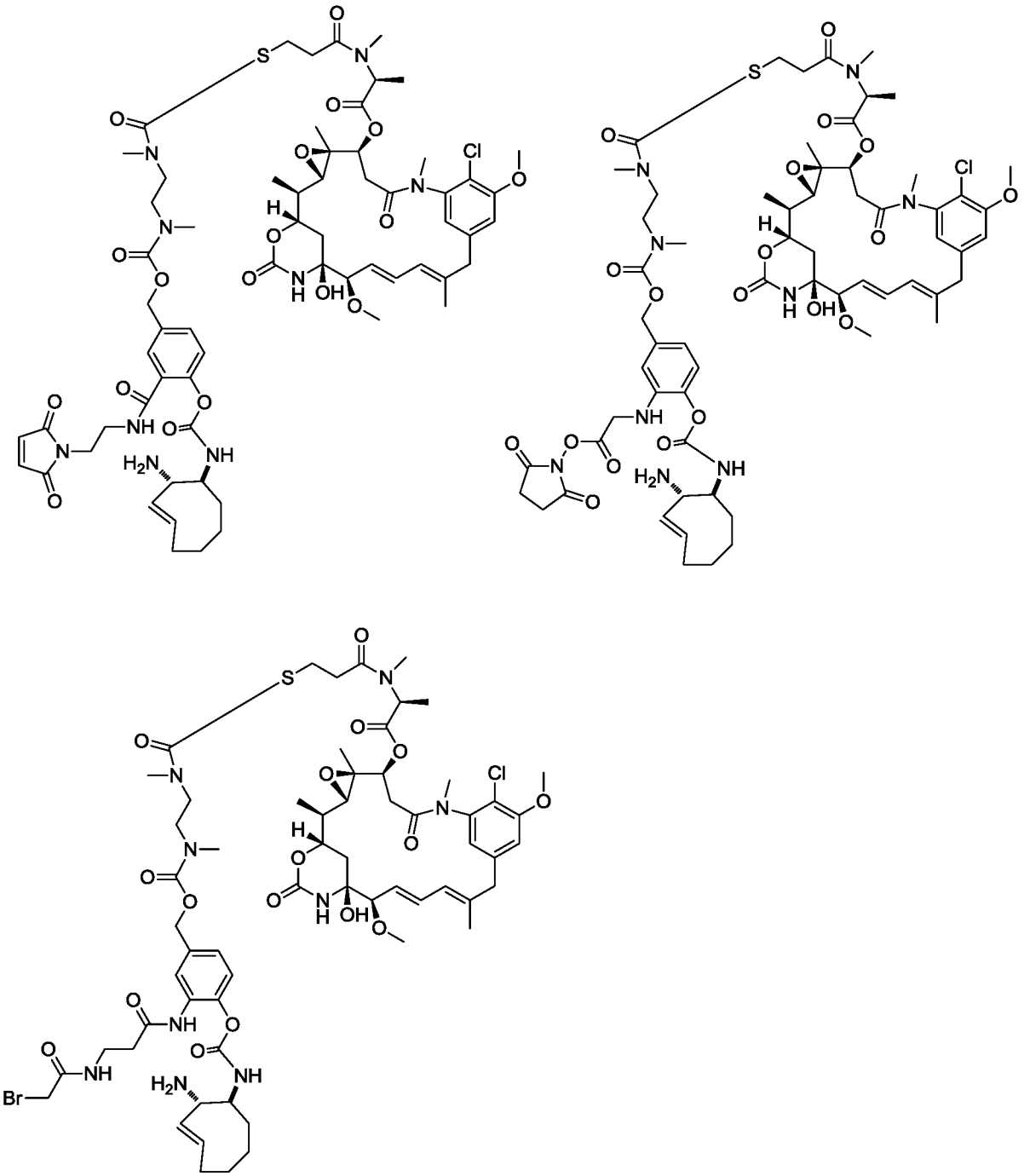
Example 14. Structures of trigger-drug constructs that can be conjugated to a targeting agent T^T eg via an amine or thiol moiety.

Auristatin E (MMAE) toxin is attached via a self immolative linker L^D to a TCO trigger and via S^P to a reactive moiety for T^T conjugation.



Example 15. Structures of trigger-drug constructs that can be conjugated to a targeting agent T^T eg via an amine or thiol moiety.

Maytansine toxin is attached via a self immolative linker L^D to a TCO trigger and via S^P to a reactive moiety for T^T conjugation.



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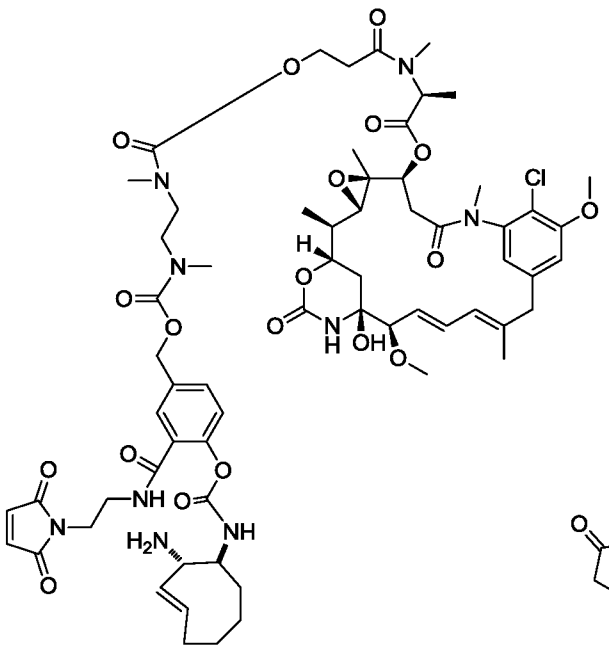
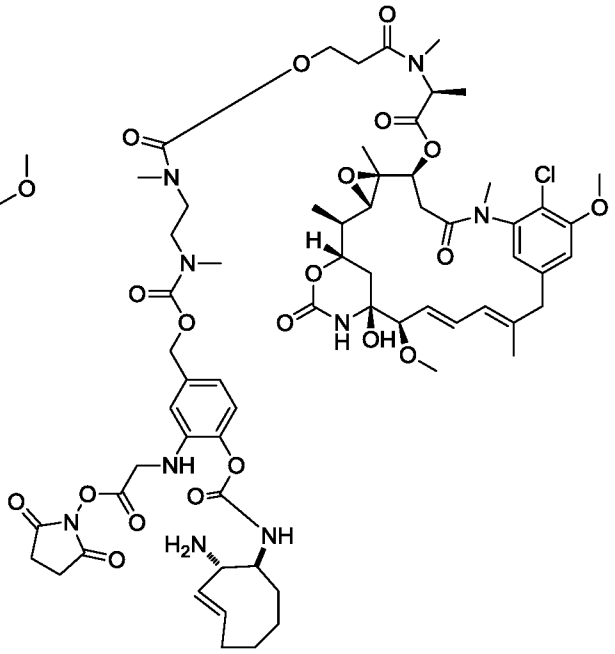
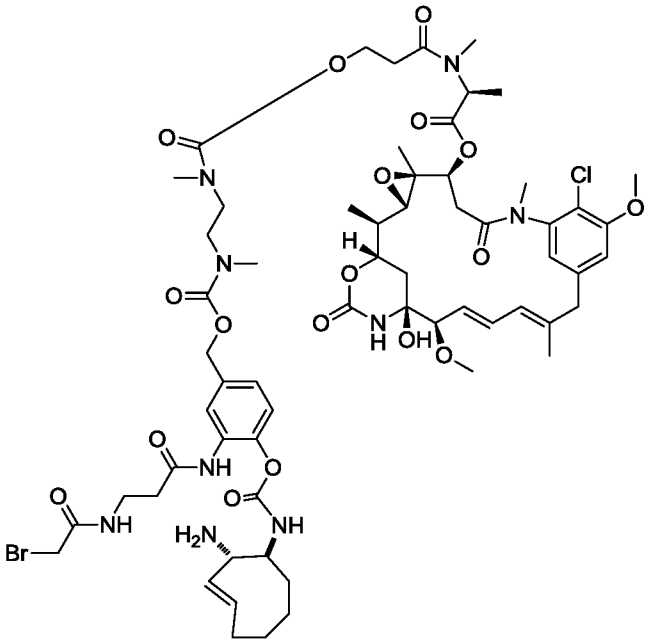
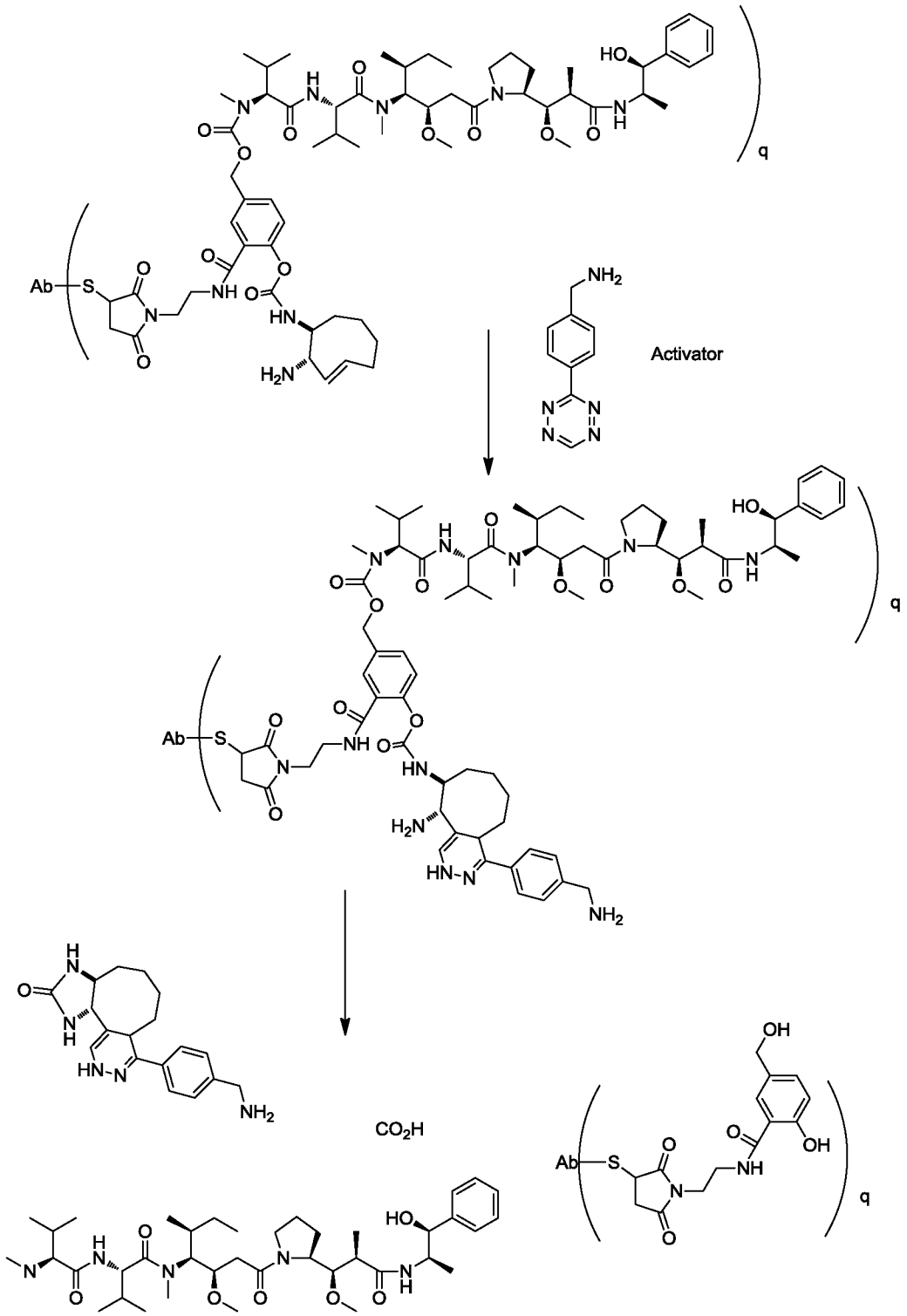






Example 16. Activation of tumor bound CC49-Auristatin E conjugate.

CC49 as mAb or mAb fragment binds the non-internalizaling pan-solid tumor marker TAG72. After Prodrug administration, tumor binding and clearance from blood, the Activator is injected. The reaction of the Activator with the TCO trigger in the Prodrug results in release of Auristatin E from CC49 (antibody, or antibody fragment), allowing it to penetrate the cancer cell inside which it has its anticancer action.



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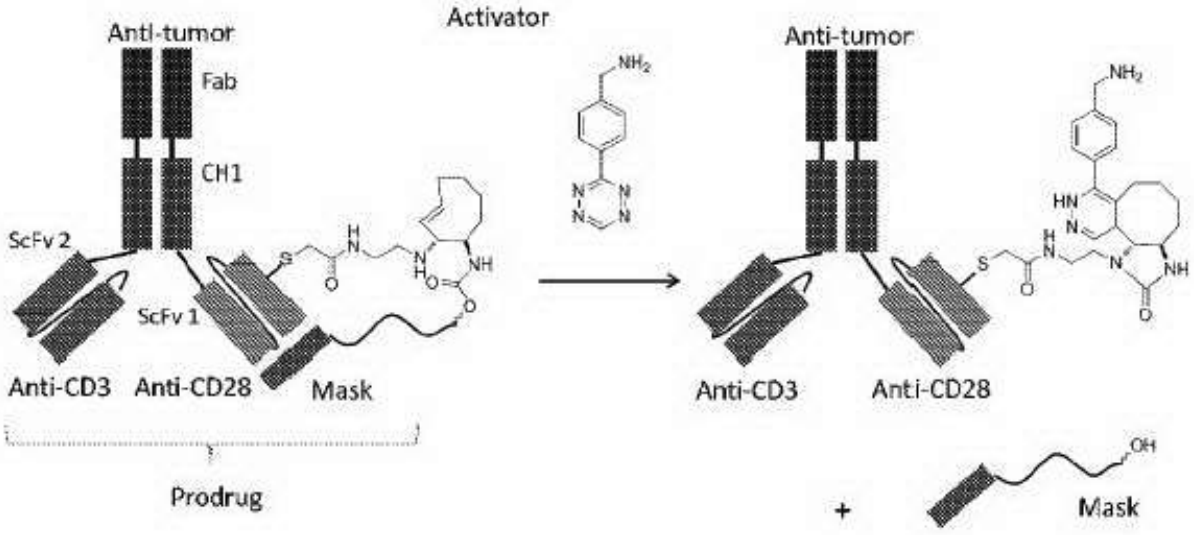
Example 17. Activation of tumor-bound T-cell engaging triabody.

The triabody comprises a tumor-binding moiety, a CD3 T-cell engaging moiety, and a CD28 T-cell co-stimulatory moiety. As the CD3 and CD28 combined in one molecule will result in unacceptable toxic effect off target, the anti-CD28 domain is blocked by a Masking Moiety M^M, a peptide resembling the CD28 binding domain and which has affinity for the anti-CD28 moiety. This peptide is linked through a further peptide or a PEG chain S^P to the TCO trigger which is itself conjugated to a site specifically engineered cysteine. After Prodrug administration, tumor binding and clearance from blood, the Activator is injected. The reaction of the Activator with the TCO trigger in the Prodrug results in release of the Masking Moiety from the anti-CD28 domain enabling CD28 co-stimulation of T-cells, boosting the T-cell mediated anticancer effect, while avoiding off target toxicity.



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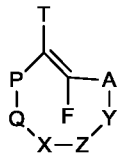
CLAIMS:

1. A kit for the administration and activation of a Prodrug, the kit comprising a Drug D^D linked, directly or indirectly, to a Trigger moiety T^R, and an Activator for the Trigger moiety, wherein the Trigger moiety comprises a dienophile and the Activator comprises a diene, the dienophile, including said Drug linked thereto, satisfying the following formula (1a):



(1a)

wherein T, F each independently denotes H, or a substituent selected from the group consisting of alkyl, F, Cl, Br or I; the meaning of the letters A,P,Q,X,Y, and Z is selected from the group consisting of the following (1) to (6):

(1) one of the bonds PQ, QP, QX, XQ, XZ, ZX, ZY, YZ, YA, AY consists of -CR^aX^D-CR^aY^D-, the remaining groups constituted by A,Y,Z,X,Q,P being independently from each other CR^a_2, S, O, SiR^b_2, such that P and A are CR^a_2, and no adjacent pairs of atoms are present selected from the group consisting of O-O, O-S, and S-S, and such that Si, if present, is adjacent to CR^a_2 or O; X^D is O-C(O)-$(L^D)_n$-(D^D), S-C(O)-$(L^D)_n$-(D^D), O-C(S)-$(L^D)_n$-(D^D), S-C(S)-$(L^D)_n$-(D^D), NR^c-C(O)-$(L^D)_n$-(D^D), NR^c-C(S)-$(L^D)_n$-(D^D); and Y^D is NHR^c, OH, SH; or X^D is C(O)-$(L^D)_n$-(D^D), C(S)-$(L^D)_n$-(D^D); and Y^D is $CR^c_2NHR^c$, CR^c_2OH, CR^c_2SH, NH-NH_2, O-NH_2, or NH-OH;

(2) A is CR^aX^D and Z is CR^aY^D, or Z is CR^aX^D and A is CR^aY^D, or P is CR^aX^D and X is CR^aY^D, or X is CR^aX^D and P is CR^aY^D, such that X^D and Y^D are positioned in a *trans* conformation with respect to one another; the remaining groups constituted by A,Y,Z,X,Q, and P being independently from each other CR^a_2, S, O, SiR^b_2, such that P and A are CR^a_2, and no adjacent pairs of atoms are present selected from the group consisting of O-O, O-S, and S-S, and such that Si, if present, is adjacent to CR^a_2 or O; X^D is O-C(O)-$(L^D)_n$-(D^D), S-C(O)-$(L^D)_n$-(D^D), O-C(S)-$(L^D)_n$-(D^D), S-C(S)-$(L^D)_n$-(D^D), NR^c-C(O)-$(L^D)_n$-(D^D), NR^c-C(S)-$(L^D)_n$-(D^D), and Y^D is NHR^c, OH, SH, $CR^c_2NHR^c$, CR^c_2OH, CR^c_2SH, NH-NH_2, O-NH_2, or NH-OH; or X^D is CR^c_2-O-C(O)-$(L^D)_n$-(D^D), CR^c_2-S-C(O)-$(L^D)_n$-(D^D), CR^c_2-O-C(S)-$(L^D)_n$-(D^D), CR^c_2-S-C(S)-$(L^D)_n$-(D^D), CR^c_2-NR^c-C(O)-$(L^D)_n$-(D^D), CR^c_2-NR^c-C(S)-$(L^D)_n$-(D^D), and Y^D is NHR^c, OH, SH; or X^D is C(O)-$(L^D)_n$-(D^D), C(S)-$(L^D)_n$-(D^D); and Y^D is $CR^c_2NHR^c$, CR^c_2OH, CR^c_2SH, NH-NH_2, O-NH_2, NH-OH.

(3) A is CR^aY^D and one of P, Q, X, Z is CR^aX^D, or P is CR^aY^D and one of A, Y, Z, X is CR^aX^D, or Y is CR^aY^D and X or P is CR^aX^D, or Q is CR^aY^D and Z or A is CR^aX^D, or either Z or X is CR^aY^D and A or P is CR^aX^D, such that X^D and Y^D are positioned in a *trans* conformation with respect to one another; the remaining groups constituted by A,Y,Z,X,Q, and P being independently from each other CR^a_2, S, O, SiR^b_2, such that P and A are CR^a_2, and no adjacent pairs of atoms are present selected from the group consisting of O-O, O-S, and S-S, and such that Si, if present, is adjacent to CR^a_2 or O; X^D is $(O\text{-}C(O))_p\text{-}(L^D)_n\text{-}(D^D)$, $S\text{-}C(O)\text{-}(L^D)_n\text{-}(D^D)$, $O\text{-}C(S)\text{-}(L^D)_n\text{-}(D^D)$, $S\text{-}C(S)\text{-}(L^D)_n\text{-}(D^D)$, and Y^D is $CR^c_2NHR^c$, CR^c_2OH, CR^c_2SH, $NH\text{-}NH_2$, $O\text{-}NH_2$, NH-OH; with p being 0 or 1;

(4) P is CR^aY^D and Y is CR^aX^D, or A is CR^aY^D and Q is CR^aX^D, or Q is CR^aY^D and A is CR^aX^D, or Y is CR^aY^D and P is CR^aX^D, such that X^D and Y^D are positioned in a *trans* conformation with respect to one another; the remaining groups constituted from A,Y,Z,X,Q, and P being independently from each other CR^a_2, S, O, SiR^b_2, such that P and A are CR^a_2, and no adjacent pairs of atoms are present selected from the group consisting of O-O, O-S, and S-S, and such that Si, if present, is adjacent to CR^a_2 or O; X^D is $(O\text{-}C(O))_p\text{-}(L^D)_n\text{-}(D^D)$, $S\text{-}C(O)\text{-}(L^D)_n\text{-}(D^D)$, $O\text{-}C(S)\text{-}(L^D)_n\text{-}(D^D)$, $S\text{-}C(S)\text{-}(L^D)_n\text{-}(D^D)$; Y^D is NHR^c, OH, SH; p = 0 or 1.

(5) Y is Y^D and P is CR^aX^D, or Q is Y^D and A is CR^aX^D; the remaining groups constituted by A,Y,Z,X,Q, and P being independently from each other CR^a_2, S, O, SiR^b_2, such that P and A are CR^a_2, and no adjacent pairs of atoms are present selected from the group consisting of O-O, O-S, and S-S, and such that Si, if present, is adjacent to CR^a_2 or O; X^D is $(O\text{-}C(O))_p\text{-}(L^D)_n\text{-}(D^D)$, $S\text{-}C(O)\text{-}(L^D)_n\text{-}(D^D)$, $O\text{-}C(S)\text{-}(L^D)_n\text{-}(D^D)$, $S\text{-}C(S)\text{-}(L^D)_n\text{-}(D^D)$, $NR^c\text{-}C(O)\text{-}(L^D)_n\text{-}(D^D)$, $NR^c\text{-}C(S)\text{-}(L^D)_n\text{-}(D^D)$, $C(O)\text{-}(L^D)_n\text{-}(D^D)$, $C(S)\text{-}(L^D)_n\text{-}(D^D)$; Y^D is NH; p = 0 or 1;

(6) Y is Y^D and P or Q is X^D, or Q is Y^D and A or Y is X^D; the remaining groups constituted by A,Y,Z,X,Q, and P being independently from each other CR^a_2, S, O, SiR^b_2, such that P and A are CR^a_2, and no adjacent pairs of atoms are present selected from the group consisting of O-O, O-S, and S-S, and such that Si, if present, is adjacent to CR^a_2 or O; X^D is $N\text{-}C(O)\text{-}(L^D)_n\text{-}(D^D)$, $N\text{-}C(S)\text{-}(L^D)_n\text{-}(D^D)$; Y^D is NH;

wherein each R^a independently is selected from the group consisting of H, alkyl, aryl, OR', SR', S(=O)R''', $S(=O)_2R'''$, $S(=O)_2NR'R''$, Si-R''', Si-O-R''', OC(=O)R''', SC(=O)R''', OC(=S)R''', SC(=S)R''', F, Cl, Br, I, N_3, SO_2H, SO_3H, SO_4H, PO_3H, PO_4H, NO, NO_2, CN, OCN, SCN, NCO, NCS, CF_3, CF_2-R', NR'R", C(=O)R', C(=S)R', C(=O)O-R', C(=S)O-R', C(=O)S-R', C(=S)S-R', C(=O)NR'R", C(=S)NR'R'', NR'C(=O)-R''', NR'C(=S)-R''',

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NR'C(=O)O-R''', NR'C(=S)O-R''', NR'C(=O)S-R''', NR'C(=S)S-R''', OC(=O)NR'-R''', SC(=O)NR'-R''', OC(=S)NR'-R''', SC(=S)NR'-R''', NR'C(=O)NR"-R'', NR'C(=S)NR"-R'', CR'NR'', with each R' and each R'' independently being H, aryl or alkyl and R''' independently being aryl or alkyl; wherein each R^b is independently selected from the group consisting of H, alkyl, aryl, O-alkyl, O-aryl, OH; wherein each R^c is independently selected from H, C_{1-6} alkyl and C_{1-6} aryl;

wherein two or more $R^{a,b,c}$ moieties together may form a ring;

$(L^D)_n$ is an optional linker with n= 0 or 1, preferably linked to T^R via S, N, NH, or O, wherein these atoms are part of the linker, which may consist of multiple units arranged linearly and/or branched; D^D is one or more drugs, preferably linked via S, N, NH, or O, wherein these atoms are part of the therapeutic moiety.

2. A kit according to claim 1, wherein A,P,Q,X,Y, and Z are selected such that one of the bonds PQ, QP, QX, XQ, XZ, ZX, ZY, YZ, YA, AY consists of $-CR^aX^D-CR^aY^D-$, the remaining groups constituted by A,Y,Z,X,Q,P being independently from each other CR^a_2, S, O, SiR^b_2, such that P and A are CR^a_2, and no adjacent pairs of atoms are present selected from the group consisting of O-O, O-S, and S-S, and such that Si, if present, is adjacent to CR^a_2 or O; X^D is O-C(O)-$(L^D)_n$-(D^D), S-C(O)-$(L^D)_n$-(D^D), O-C(S)-$(L^D)_n$-(D^D), S-C(S)-$(L^D)_n$-(D^D), NR^c-C(O)-$(L^D)_n$-(D^D), NR^c-C(S)-$(L^D)_n$-(D^D); and Y^D is NHR^c, OH, SH; or X^D is C(O)-$(L^D)_n$-(D^D), C(S)-$(L^D)_n$-(D^D); and Y^D is $CR^c_2NHR^c$, CR^c_2OH, CR^c_2SH, $NH-NH_2$, $O-NH_2$, or NH-OH.

3. A kit according to claim 2, wherein PQ, QP, AY or YA is $-CR^aX^D-CR^aY^D-$, and X^D and Y^D are positioned *trans* relative to each other.

4. A kit according to claim 2, wherein ZX or XZ is $-CR^aX^D-CR^aY^D$ -, and X^D and Y^D are positioned *cis* relative to each other.

5. A kit according to any one of the preceding claims, wherein X^D is NR^c-C(O)-$(L^D)_n$-(D^D), and Y^D is NHR^c.

6. A kit according to claim 5, wherein the dienophile is a compound selected from the following structures:

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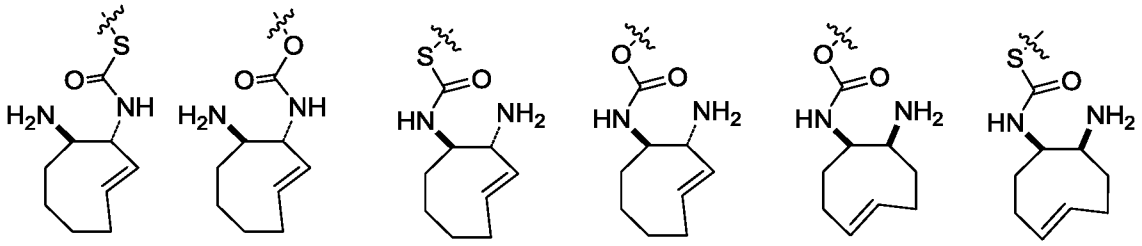
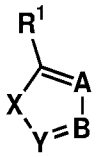


〰 = rest of attached D^D, L^D-D^D, optionally comprising T^T or S^P-T^T or M^M or S^P-M^M

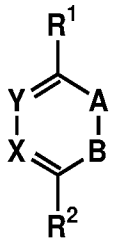
(wherein the abbreviation T^T stands for a targeting agent, S^P for a spacer, and M^M for a masking moiety).

7. A kit according to any one of the preceding claims, wherein the activator comprises a diene selected from the dienes, according to formulae (2)-(4):



(2)

wherein R^1 is selected from the group consisting of H, alkyl, aryl, CF_3, CF_2-R', OR', SR', C(=O)R', C(=S)R', C(=O)O-R', C(=O)S-R', C(=S)O-R', C(=S)S-R'', C(=O)NR'R'', C(=S)NR'R'', NR'R'', NR'C(=O)R'', NR'C(=S)R'', NR'C(=O)OR'', NR'C(=S)OR'', NR'C(=O)SR'', NR'C(=S)SR'', NR'C(=O)NR''R'', NR'C(=S)NR''R'' with each R' and each R'' independently being H, aryl or alkyl; A and B each independently are selected from the group consisting of alkyl-substituted carbon, aryl substituted carbon, nitrogen, N^+O^-, N^+R with R being alkyl, with the proviso that A and B are not both carbon; X is selected from the group consisting of O, N-alkyl, and C=O, and Y is CR with R being selected from the group consisting of H, alkyl, aryl, C(=O)OR', C(=O)SR', C(=S)OR', C(=S)SR', C(=O)NR'R'' with R' and R'' each independently being H, aryl or alkyl;



(3)

wherein R^1 and R^2 each independently are selected from the group consisting of H, alkyl, aryl, CF_3, CF_2-R', NO_2, OR', SR', C(=O)R', C(=S)R', OC(=O)R''', SC(=O)R''',

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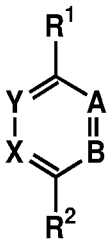
$OC(=S)R'''$, $SC(=S)R'''$, $S(=O)R'$, $S(=O)_2R'''$, $S(=O)_2NR'R''$, $C(=O)O\text{-}R'$, $C(=O)S\text{-}R'$, $C(=S)O\text{-}R'$, $C(=S)S\text{-}R'$, $C(=O)NR'R''$, $C(=S)NR'R''$, $NR'R''$, $NR'C(=O)R''$, $NR'C(=S)R''$, $NR'C(=O)OR''$, $NR'C(=S)OR''$, $NR'C(=O)SR''$, $NR'C(=S)SR''$, $OC(=O)NR'R''$, $SC(=O)NR'R''$, $OC(=S)NR'R''$, $SC(=S)NR'R''$, $NR'C(=O)NR''R''$, $NR'C(=S)NR''R''$ with each R' and each R'' independently being H, aryl or alkyl, and R''' independently being aryl or alkyl; A is selected from the group consisting of N-alkyl, N-aryl, C=O, and CN-alkyl; B is O or S; X is selected from the group consisting of N, CH, C-alkyl, C-aryl, $CC(=O)R'$, $CC(=S)R'$, $CS(=O)R'$, $CS(=O)_2R'''$, $CC(=O)O\text{-}R'$, $CC(=O)S\text{-}R'$, $CC(=S)O\text{-}R'$, $CC(=S)S\text{-}R'$, $CC(=O)NR'R''$, $CC(=S)NR'R''$, R' and R'' each independently being H, aryl or alkyl and R''' independently being aryl or alkyl; Y is selected from the group consisting of CH, C-alkyl, C-aryl, N, and N^+O^-;



(4)

wherein R^1 and R^2 each independently are selected from the group consisting of H, alkyl, aryl, CF_3, $CF_2\text{-}R'$, NO, NO_2, OR', SR', CN, $C(=O)R'$, $C(=S)R'$, $OC(=O)R'''$, $SC(=O)R'''$, $OC(=S)R'''$, $SC(=S)R'''$, $S(=O)R'$, $S(=O)_2R'''$, $S(=O)_2OR'$, $PO_3R'R"$, $S(=O)_2NR'R''$, $C(=O)O\text{-}R'$, $C(=O)S\text{-}R'$, $C(=S)O\text{-}R'$, $C(=S)S\text{-}R'$, $C(=O)NR'R''$, $C(=S)NR'R''$, $NR'R''$, $NR'C(=O)R''$, $NR'C(=S)R''$, $NR'C(=O)OR''$, $NR'C(=S)OR''$, $NR'C(=O)SR''$, $NR'C(=S)SR''$, $OC(=O)NR'R''$, $SC(=O)NR'R''$, $OC(=S)NR'R''$, $SC(=S)NR'R''$, $NR'C(=O)NR''R''$, $NR'C(=S)NR''R''$ with each R' and each R'' independently being H, aryl or alkyl, and R''' independently being aryl or alkyl; A is selected from the group consisting of N, C-alkyl, C-aryl, and N^+O^-; B is N; X is selected from the group consisting of N, CH, C-alkyl, C-aryl, $CC(=O)R'$, $CC(=S)R'$, $CS(=O)R'$, $CS(=O)_2R'''$, $CC(=O)O\text{-}R'$, $CC(=O)S\text{-}R'$, $CC(=S)O\text{-}R'$, $CC(=S)S\text{-}R'$, $CC(=O)NR'R''$, $CC(=S)NR'R''$, R' and R'' each independently being H, aryl or alkyl and R''' independently being aryl or alkyl; Y is selected from the group consisting of CH, C-alkyl, C-aryl, N, and N^+O^-;.

8. A kit according to claim 7, wherein the diene satisfies formula (7)

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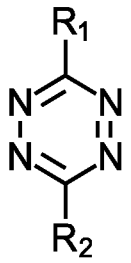
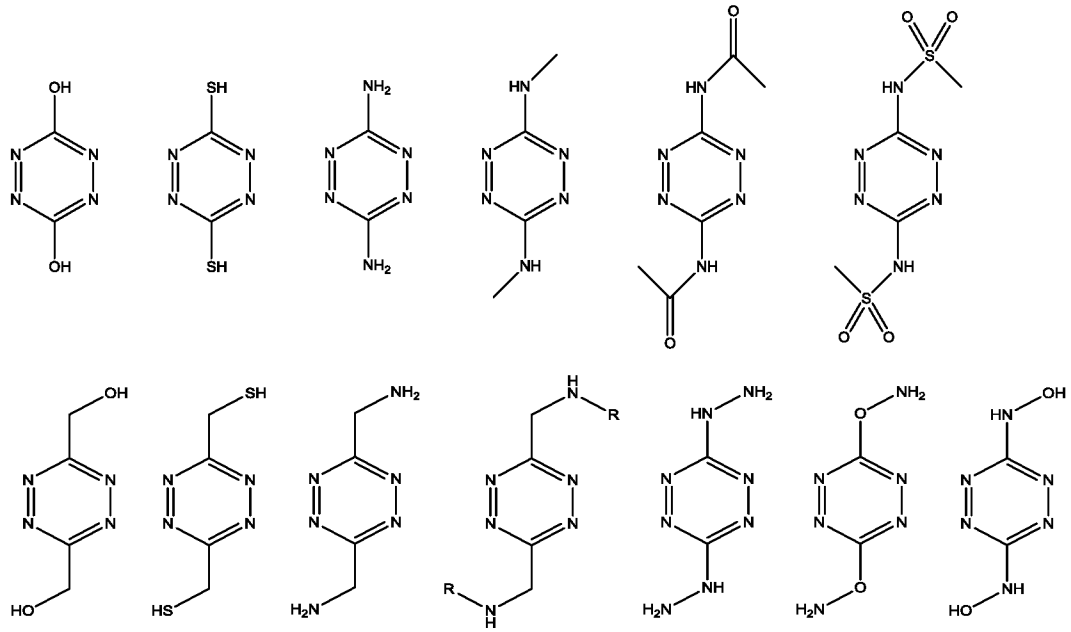


, being a tetrazine *para* substituted with R_1 and R_2, wherein R_1 and R_2 each independently denote a substituent selected from the group consisting of H, alkyl, NO_2, CF_3, CN, COOR, CONHR, $CONR_2$, COR, SO_2R, SO_2OR, SO_2NR_2, PO_3R_2, NO, 2-pyridyl, 3-pyridyl, 4-pyridyl, 2,6-pyrimidyl, 3,5-pyrimidyl, 2,4-pyrimidyl, 2,4 imidazyl, 2,5 imidazyl and phenyl, optionally substituted with one or more electron-withdrawing groups selected from the group consisting of NO_2, F, Cl, CF_3, CN, COOR, CONHR, CONR, COR, SO_2R, SO_2OR, SO_2NR_2, PO_3R_2, NO, and Ar, wherein R is H or C_1-C_6 alkyl, and Ar stands for phenyl, pyridyl, or naphthyl.

9. A kit according to claim 7, wherein the diene is selected from the group consisting of:



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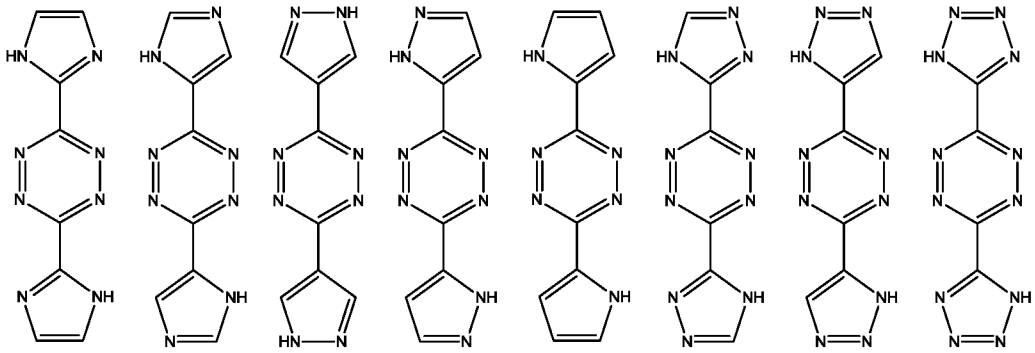
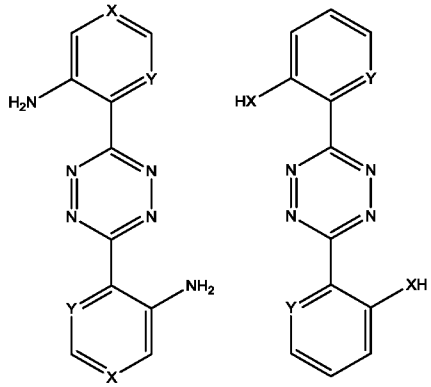
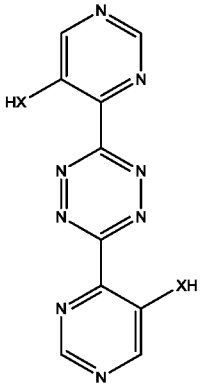


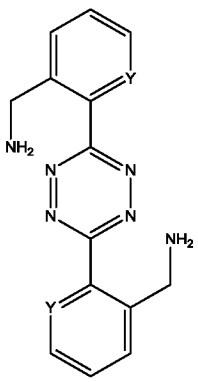


both Y = C and both X = C, or
both Y = C and both X = N, or
both Y = N and both X = C, or
both Y = N and both X = N

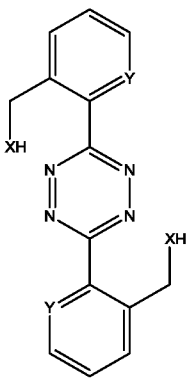
both X = O and both Y = C, or
both X = O and both Y = N, or
both X = S and both Y = C, or
both X = S and both Y = N



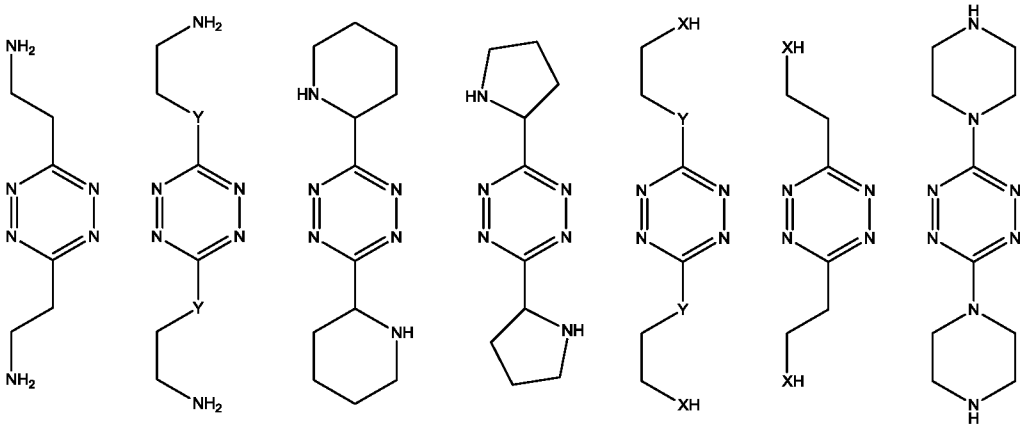
both X = O, or
both X = S



both Y = C, or
both Y = N



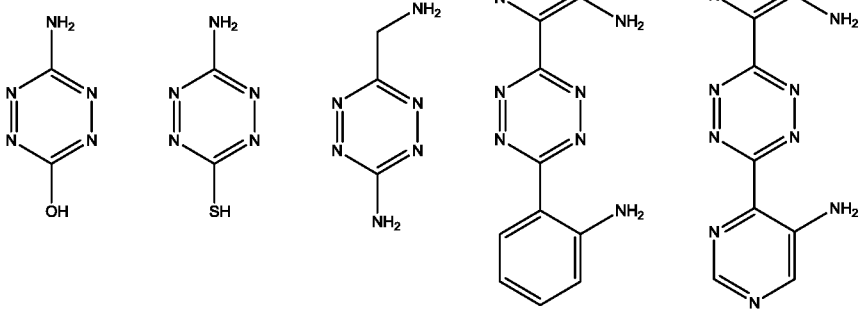
both X = O and both Y = C, or
both X = O and both Y = N, or
both X = S and both Y = C, or
both X = S and both Y = N



both Y = O, or
both Y = S

both X = O and both Y = O, or
both X = O and both Y = S, or
both X = S and both Y = O, or
both X = S and both Y = S

both X = O, or
both X = S



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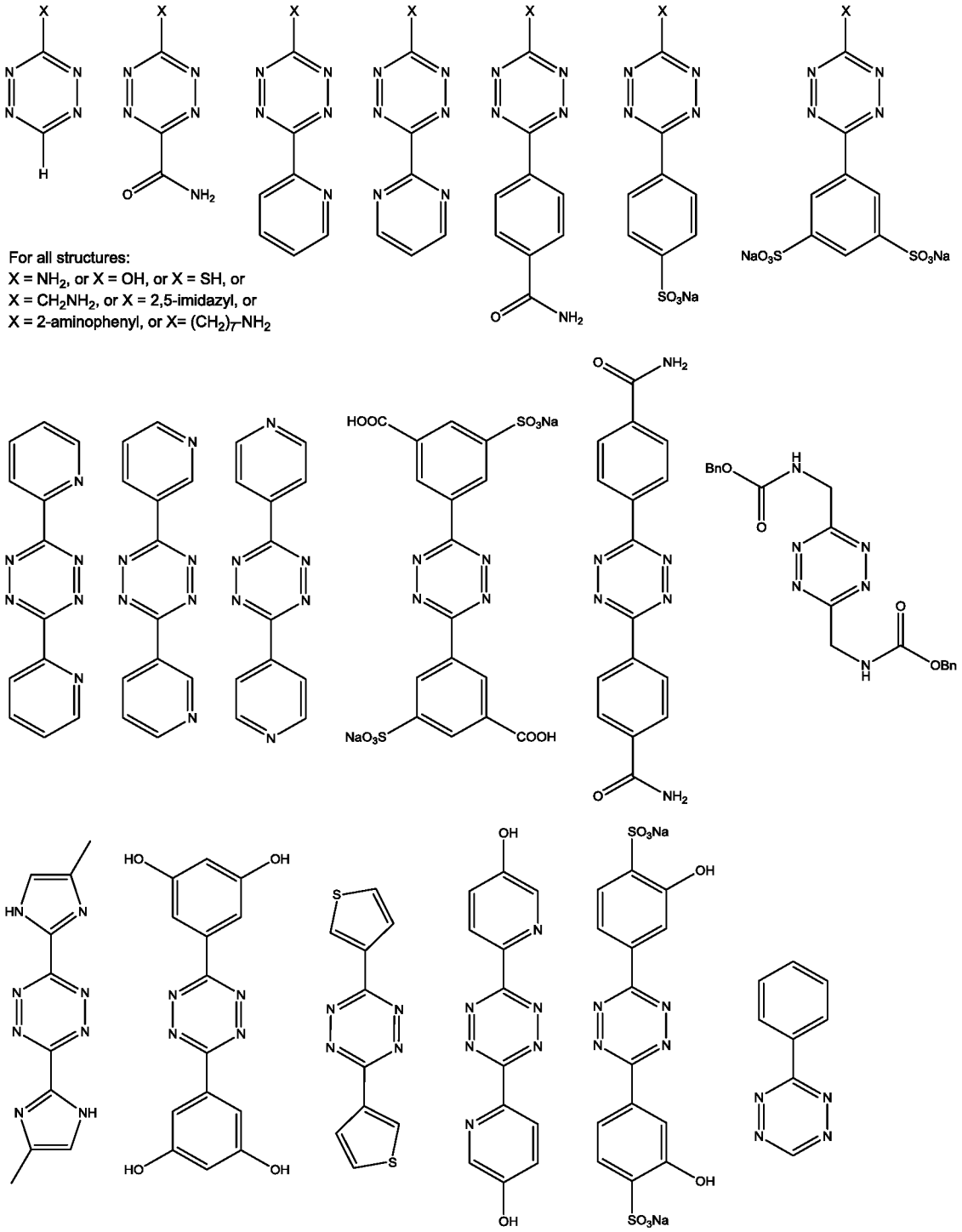

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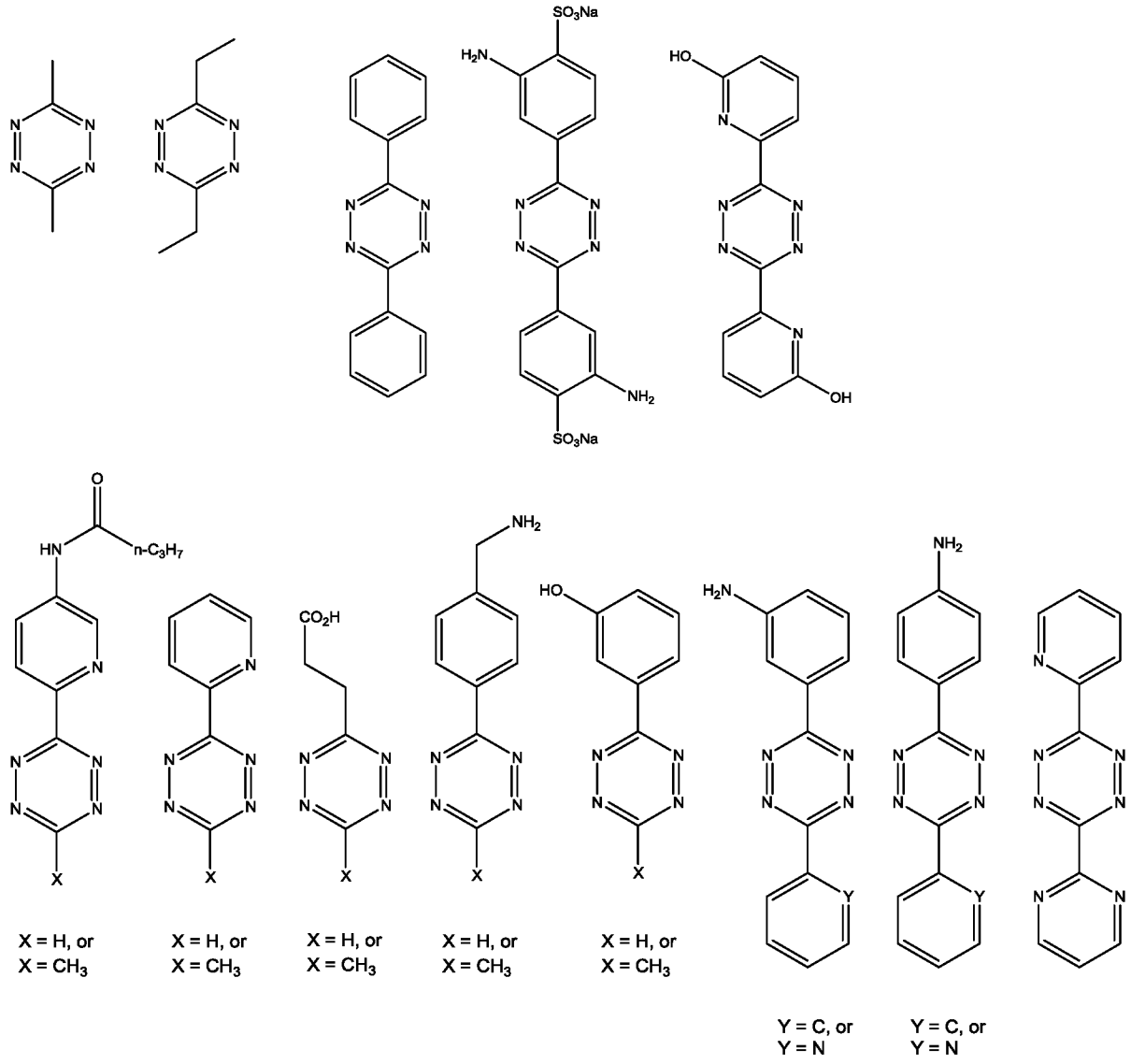

10. A kit according to claim 7, wherein the diene is selected from the group consisting of:

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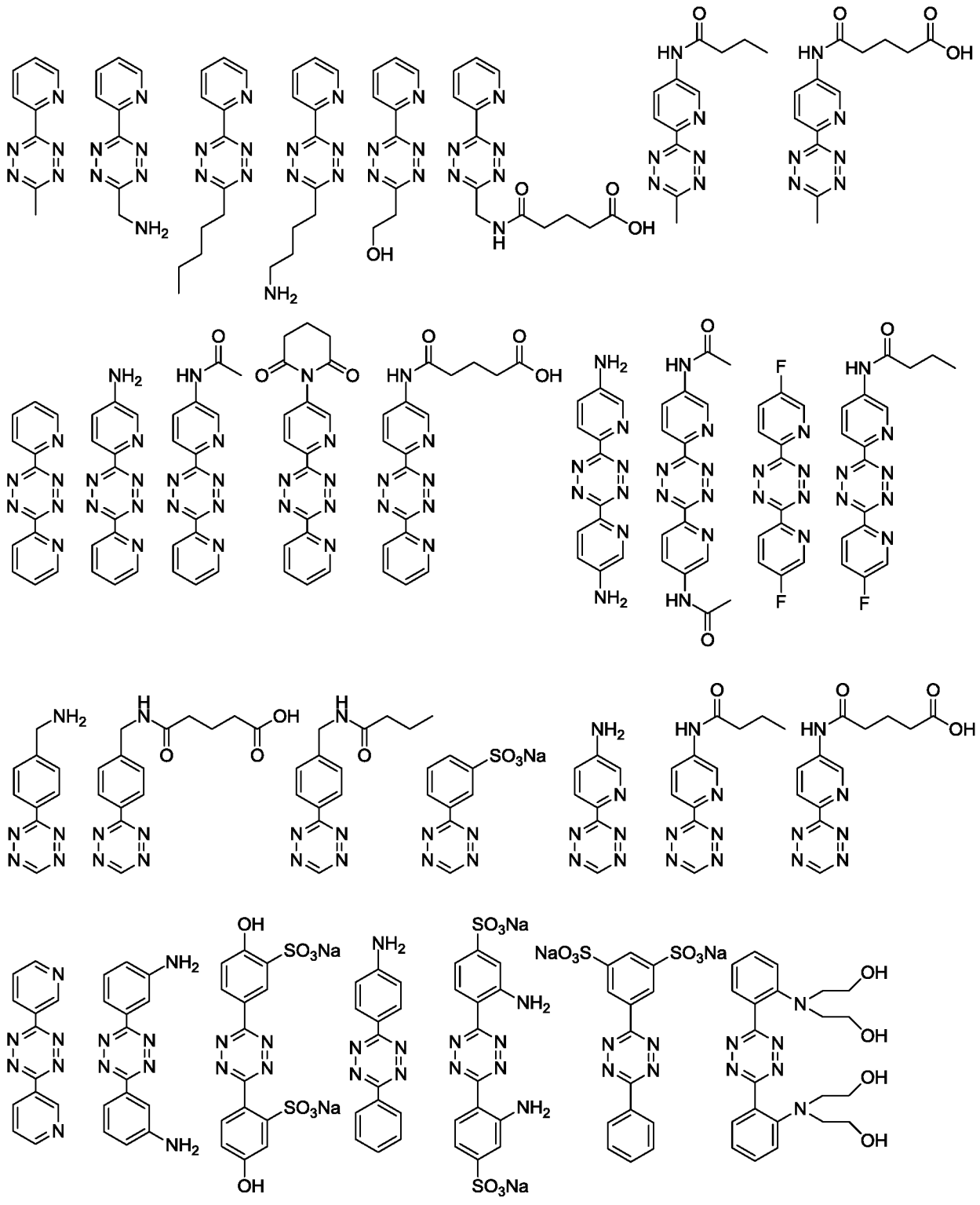

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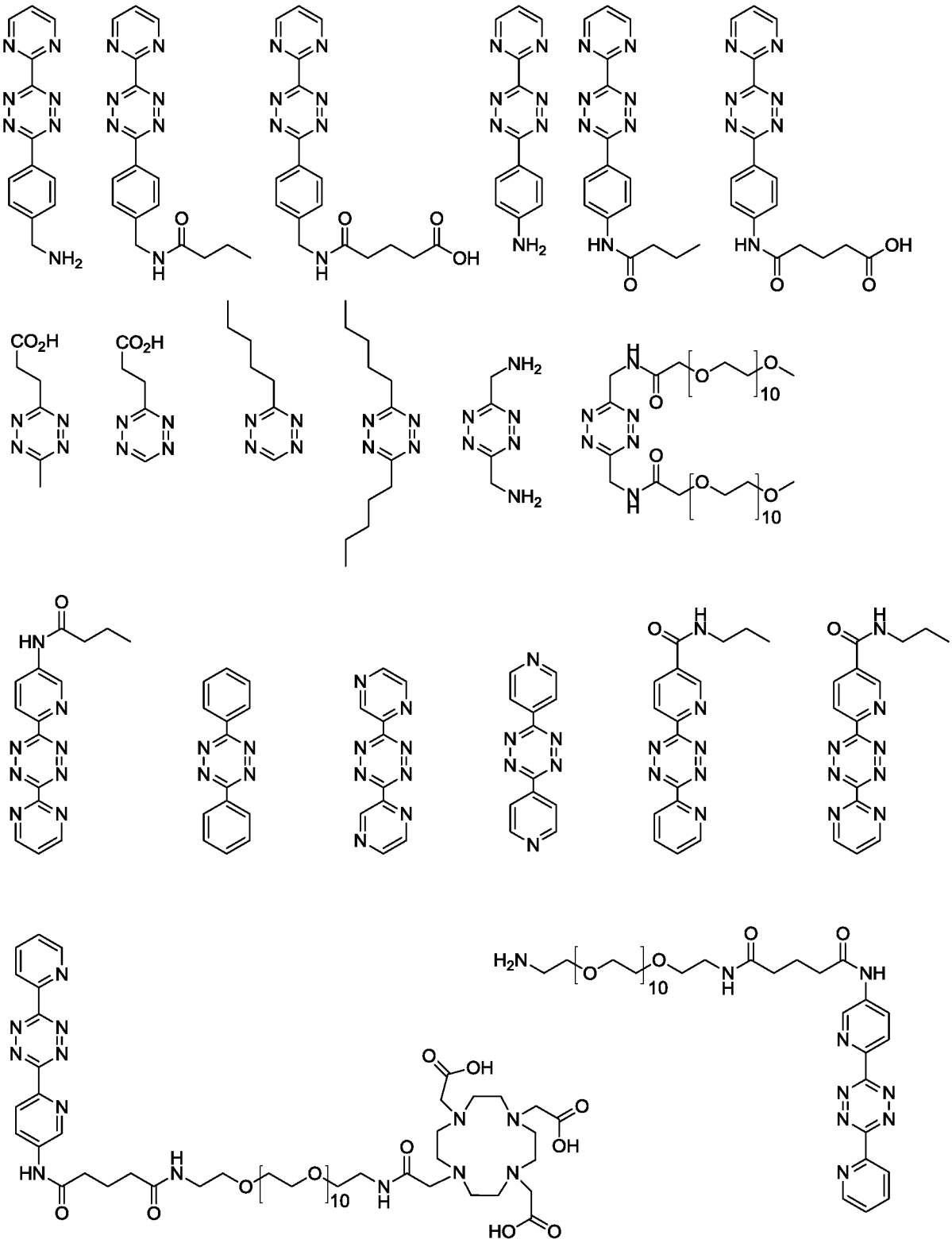

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11. A kit according to any one of the preceding claims, wherein at least one of the drug D^D or the linker L^D or the trigger moiety T^R comprises a targeting agent T^T, preferably an antibody.

12. A kit according to any one of the claims 1 to 10, wherein at least one of the L^D or the trigger moiety T^R comprises a masking moiety M^M, preferably a peptide.

13. A kit according to any one of the preceding claims, wherein the drug is a T-cell engaging antibody construct.

14. A kit according to any one of the claims 1 to 11, wherein the Prodrug comprises an antibody-toxin or antibody-drug conjugate.

15. A prodrug comprising a drug compound linked, directly or indirectly, to a dienophile moiety of formula (1a), as defined in any one of the claims 1-6.

16. A method of modifying a drug compound into a prodrug that can be triggered by an abiotic, bio-orthogonal reaction, comprising providing a drug and chemically linking the drug to a dienophile moiety, so as to form a prodrug of formula (1a) as defined in any one of the claims 1-6.

17. A method of treatment wherein a patient suffering from a disease that can be modulated by a drug, is treated by administering, to said patient, a prodrug comprising a trigger moiety and an activator for the trigger moiety after activation of which the drug will be released, wherein the trigger moiety comprises a *trans*-cyclooctene ring, the ring optionally including one or more hetero-atoms, and the activator comprises a diene being selected so as to be capable of reacting with the dienophile in an inverse electron-demand Diels-Alder reaction, the trigger moiety satisfying the formula (1a) as defined in any one of the claims 1 to 6.

18. A compound satisfying the formula (1a) as defined in any one of the claims 1 to 6:

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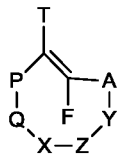

(1a)

said compound comprising a linkage to a drug, for use in prodrug therapy in an animal or a human being.

19. The use of a tetrazine as an activator for the release, in a physiological environment, of a substance linked to a compound satisfying formula (1a) as defined in any one of claims 1 to 6.

20. The use of the inverse electron-demand Diels-Alder reaction between a compound satisfying formula (1a) as defined in any one of the claims 1 to 6 and a tetrazine as a chemical tool for the release, in a physiological environment, of a substance administered in a chemically bound form, wherein the substance is bound to a compound satisfying formula (1a).

21. The use of a *trans*-cyclooctene satisfying formula (1a), as defined in any one of the claims 1 to 6, as a carrier for a therapeutic compound.