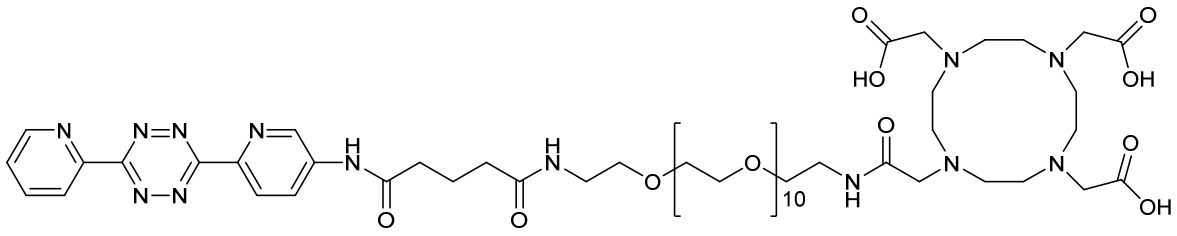
TETRAZINES WITH IMPROVED PROPERTIES

Technological field

The disclosure relates to tetrazines with improved properties. Compositions and combinations comprising the tetrazines of the disclosure, as well as methods for using and making same are provided as well.

Background

In the field of bioorthogonal chemistry the ligation between tetrazines and dienophiles, in particular *trans*-cyclooctenes, has been studied in depth. Rossin *et al.*, Angew. Chem. Int. Ed. 2010, volume 49, pages 3375-3378 describes a tetrazine refered to herein as compound **2.1** having a structure as follows:



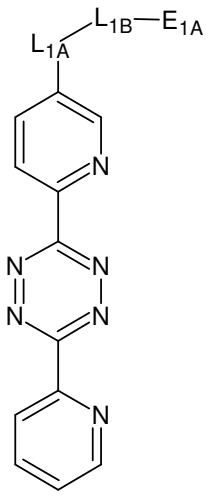
Compound **2.1** and some of its properties were described in further papers on pretargeting, viz. Rossin *et al.*, J. Nucl. Med. 2013, volume 54; pages 1989-1995; Rossin *et al.*, Bioconjugate Chem. 2013, volume 24, pages 1210-1217; Rossin *et al.*, Mol. Pharm. 2014, volume 11, pages 3090-3096; Van Duijnhoven *et al.*, J. Nucl. Med. 2015, volume 56, pages 1422-1428; and Edem *et al.* Molecules 2020, volume 25, page 463.

While the ligation works well both *in vitro* as *in vivo*, identifying new optimal compounds for clinical use remains a research focus. Along these lines it is desired to identify new tetrazines with overall good *in vitro* and *in vivo* properties, *e.g.* one or more of: good stability, good reactivity with and/or high payload release from *trans*-cyclooctenes (especially *in vivo*), low membrane permeability, low cell toxicity, and low genotoxicity.

There is thus a need for new tetrazines that address one or more of the abovementioned problems and/or desires.

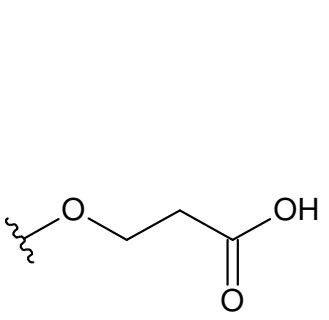
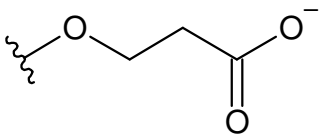
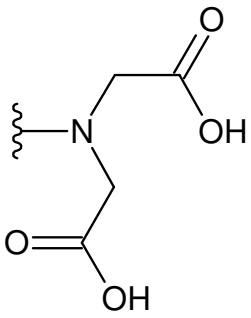
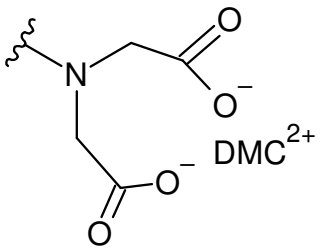
Summary

In one aspect, the disclosure relates to a compound or a salt, hydrate, or solvate thereof; wherein said compound has a structure according to Formula (1):

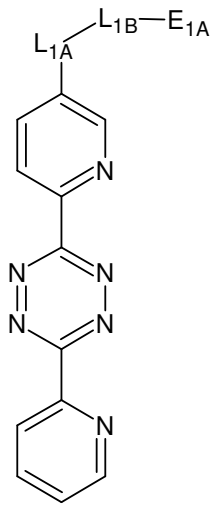


Formula (1); wherein

L_{1A} and L_{1B} are independently selected linkers; E_{1A} is selected from the group consisting of:


; 
; 
; and 
; wherein MMC^+ is a monovalent metal cation; DMC^{2+} is a divalent metal cation; preferably MMC^+ is Na^+; preferably DMC^{2+} is Ca^{2+}.

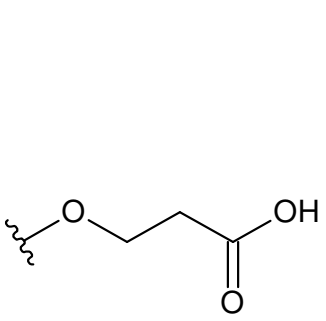
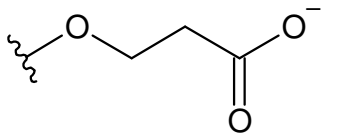
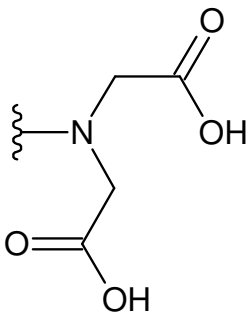
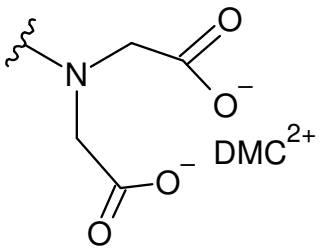
Disclosed herein is a compound or a salt, hydrate, or solvate thereof; wherein said compound has a structure according to Formula (1):



Formula (1); wherein

L_{1A} and L_{1B} are independently selected linkers;

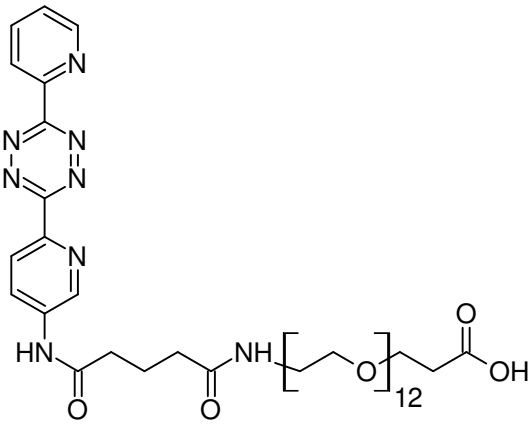
E_{1A} is selected from the group consisting of:


; 
; 
; and 
; wherein

MMC^+ is a monovalent metal cation;

DMC^{2+} is a divalent metal cation;

with the proviso that the compound of Formula (1) is not



or a salt, solvate, or hydrate thereof;

preferably MMC^{+} is Na^{+};

preferably DMC^{2+} is Ca^{2+}.

In another aspect, the disclosure pertains to a composition comprising a compound according to Formula (1), or the salt, hydrate, or solvate thereof; preferably the composition is a pharmaceutical composition.

In yet another aspect, the disclosure relates to a combination of

(A1) a compound according to Formula (1), or the salt, hydrate, or solvate thereof; and/or

(A2) a composition according to the disclosure; with

(B) a dienophile or a salt, solvate, or hydrate thereof; preferably the dienophile comprises a *trans*-cyclooctene moiety.

In a further aspect, the disclosure pertains to the compound according to Formula (1), or the salt, hydrate, or solvate thereof; the composition according to the disclosure; or the combination according to the disclosure; for use as a medicament.

In yet a further aspect, the disclosure relates to the compound according to Formula (1), or the salt, hydrate, or solvate thereof; the composition according to the disclosure; or the combination according to the disclosure; for use in the treatment of a disease in a subject; preferably the subject is a human; preferably the disease is cancer.

In a further aspect still, the disclosure pertains to a non-therapeutic method for reacting:

(ia) the compound according to Formula (1), or the salt, hydrate, or solvate thereof; and/or

(iia) the composition according to the disclosure;

with a dienophile or a salt, solvate, or hydrate thereof; wherein said method comprises the step of contacting (ia), and/or (iia) with said dienophile or salt, solvate, or hydrate thereof; preferably said non-therapeutic method is an *in vitro* method; and preferably said dienophile comprises a *trans*-cyclooctene moiety.

In yet a further aspect, the disclosure relates to a non-therapeutic use of:

(a) the compound according to Formula (1), or the salt, hydrate, or solvate thereof;

(b) the composition according to the disclosure and/or

(c) the combination according to the disclosure;

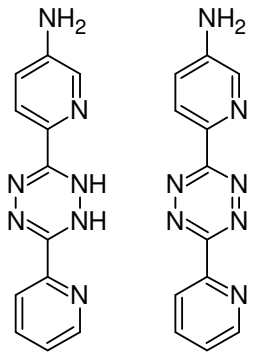
in a click reaction.

In yet a further aspect, the disclosure relates to a method for preparing a compound according to claim 7, wherein said method comprises the steps of:

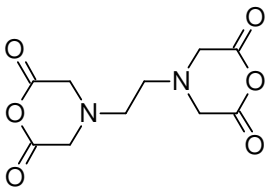
(a) reacting SM1a or SM1b with a reagent selected from the group consisting of SM2, SM3, and SM4;

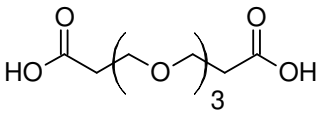
(b) if SM1a is used in step (a), subjecting the reaction product of step (a) to oxidation;

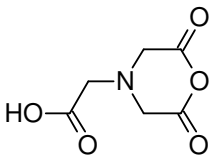
(c) optionally, subjecting the reaction product of step (a) or step (b) to salt formation; wherein SM1a, SM1b, SM2, SM3, and SM4 are:


SM1a

SM1b


SM2


SM3


SM4

;

preferably in step (a) SM1a is used; preferably in step (b) the reaction product of step (a) is contacted with sodium nitrite.

In a further aspect, the disclosure relates to a method of treating a disease in a subject, wherein said method comprises the step of administering to said subject:

(a) the compound according to Formula (1), or the salt, hydrate, or solvate thereof;

(b) the composition according to the disclosure; and/or

(c) the combination according to the disclosure; preferably the subject is a human; preferably the disease is cancer.

In a final aspect, the disclosure relates to a use of a compound of Formula (1), a composition of the disclosure, or a combination of the disclosure in the manufacture of a medicament for the treatment of a disease in a subject; preferably the subject is a human; preferably the disease is cancer.

Detailed Description

Above mentioned compound **2.1** was first described by Rossin *et al.*, Angew. Chem. Int. Ed. 2010, volume 49, pages 3375-3378. Therein, said compound was used in relation to pretargeting of tumors, and selected for its good pharmacokinetics, reactivity and sufficient stability *in vivo*.

Furthermore, compound **2.1** has also been used *in vivo* to bind and detect unreacted *trans*-cyclooctenes by Rossin *et al.*, Bioconjugate Chem. 2016, volume 27, pages 1697-1706; and Rossin *et al.*, Nature Commun. 2018, volume 9, article 1484.

Additionally, compound **2.1** has some very interesting properties for *in vivo* release of a payload, typically a drug, attached to a *trans*-cyclooctene. Compound **2.1** as a higher reactivity towards *trans*-cyclooctenes than other tetrazines and has a good clearance rate (Rossin et al., Nature Commun. 2018, volume 9, article 1484, page 6, left column). Although compound **2.1** has a low release yield with most *trans*-cyclooctenes, like most bis-(2-pyridyl)-tetrazines, the release yield for bis-(2-pyridyl)-tetrazines is (near-)quantitative when using *trans*-cyclooctenes with certain substituents (WO 2020/256546, in particular Example 5 at pages 294-296). As such, compound **2.1** is also a strong candidate for use in payload-release *in vivo*. Consequently, compound **2.1** is the best-studied tetrazine for *in vivo* use in literature, and has been reported to be a promising candidate for clinical use.

However, properties of compound **2.1** that could be improved upon have been identified by the inventors and reported on herein for the first time. Such properties may arise in certain conditions when using **2.1** *in vivo* as an activator for the payload release from *trans*-cyclooctenes, which typically requires higher doses than when using **2.1** for radioimaging and/or radiotherapy.

First, it was found that compound **2.1** strongly inhibits the physiologically relevant enzymes cyclooxygenase (COX-1), acetyl cholinesterase (ACES), monoamine oxidase (MAO-B), and calcium channel L-type, dihydropyridine. Each of these proteins is important in maintaining health in a subject, and undesired inhibition of these enzymes and/or transporter may lead to side-effects. Based on this, it is desired that tetrazines be provided that show lower inhibition for one or more of these enzymes and/or transporter.

Second, it was found that **2.1** has a maximum tolerated dose (MTD) in mice of about 39 µmol/kg. This may limit the therapeutic window in which **2.1** can be safely used *in vivo*. It is therefore also desired that new tetrazines with a higher maximum tolerated dose be provided.

Furthermore, the synthesis of **2.1** comprises multiple steps, and it is desired that tetrazines be provided that can be synthesized in fewer steps and/or in a more straightforward way.

Some aspects and embodiments of the disclosure are therefore, in a broad sense, based on the judicious insight that tetrazines of Formula (1) may meet one or more of the aforementioned desires.

In particular, the tetrazines of Formula (1) can show lower inhibition of the enzymes cyclooxygenase (COX-1), acetyl cholinesterase (ACES), monoamine oxidase (MAO-B), and/or calcium channel L-type, dihydropyridine, as shown in Example 3 herein.

Moreover, the tetrazines of Formula (1) typically have a relatively higher maximum tolerated dose in mice, as shown in Example 4 herein.

Furthermore, the tetrazines of Formula (1) can be readily synthesized in fewer steps than required for **2.1**, as shown in Example 2 herein.

Finally, the tetrazines of Formula (1) in many embodiments also show further excellent *in vitro* and *in vivo* properties, as shown in Example 5 herein.

Preferred embodiments of Formula (1) are further described below. All of these embodiments can be combined as long as they are not mutually exclusive.

Compounds of Formula (1)

The compounds of the disclosure are according to Formula (1) as defined above. For ease of reference, compounds of Formula (1) are below described as *e.g.* “compounds of the disclosure”. It will be understood that also the salt, hydrate, or solvate of said compounds are included by such a statement.

In relation to Formula (1), MMC^+ is preferably selected from the group consisting of Na^+, K^+, and Cu^+. Most preferably, MMC^+ is Na^+.

In relation to Formula (1), DMC^{2+} is preferably selected from the group consisting of Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+}, Cd^{2+}, Cr^{2+}, Co^{2+}, Fe^{2+}, Pb^{2+}, Ni^{2+}, Hg^{2+}, Sn^{2+} and Pt^{2+}. Most preferably, DMC^{2+} is Ca^{2+}.

In Formula (1), L_{1A} and L_{1B} are independently selected linkers.

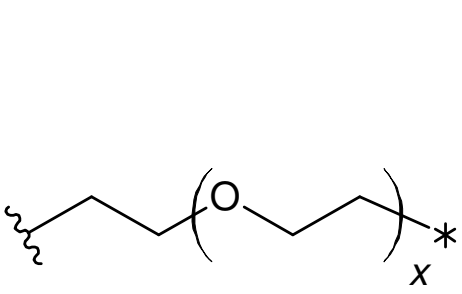
Preferably, L_{1A} is a linker containing at most 10 atoms. More preferably, L_{1A} is selected from the group consisting of -O-, -S-, -SS-, -NR_{L1A}-, -N=N-, -C(O)-, -C(O)NR_{L1A}-, -

OC(O)-, -C(O)O-, -OC(O)NR_{L1A}-, -NR_{L1A}C(O)-, -NR_{L1A}C(O)O-, -NR_{L1A}C(O)NR_4-, -SC(O)-, -C(O)S-, -SC(O)O-, -OC(O)S-, -SC(O)NR_{L1A}-, and -NR_{L1A}C(O)S-. Even more preferably, L_{1A} is -NR_{L1A}C(O)-, and most preferably L_{1A} is -NHC(O)-.

In relation to Formula (1), R_{L1A} is hydrogen or C_{1-3} alkyl. Preferably R_{L1A} is hydrogen.

Preferably, L_{1B} is a linker containing at most 50 atoms, more preferably L_{1B} is a linker containing at most 30 atoms; even more preferably L_{1B} is a linker containing at most 25 atoms; and most preferably L_{1B} is a linker containing at most 10 atoms.

Preferably, L_{1B} is selected from the group consisting of -CH_2-,



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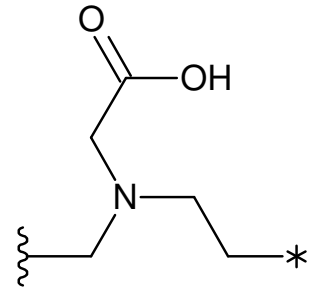

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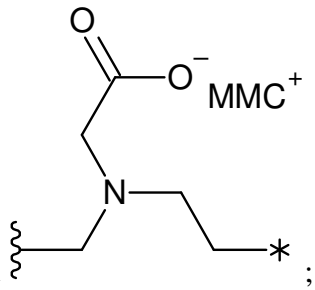
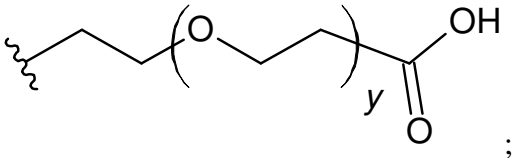


;

wherein the wiggly line indicates a bond to L_{1A} and the asterisk a bond to E_{1A}.

In relation to L_{1B} x is an integer in a range of from 1 to 12; preferably x is an integer in a range of from 2 to 7; and more preferably x is an integer in a range of from 2 to 4. Most preferably, x is 3.

Preferably, L_{1B} and E_{1A} together form a moiety selected from the group consisting of:



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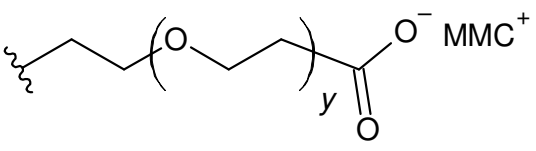

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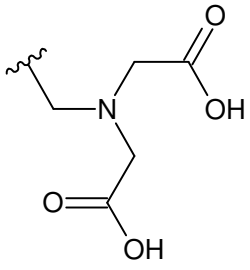

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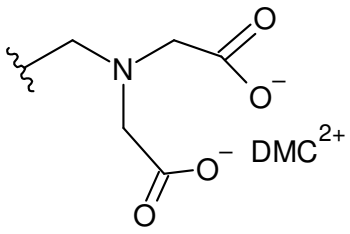

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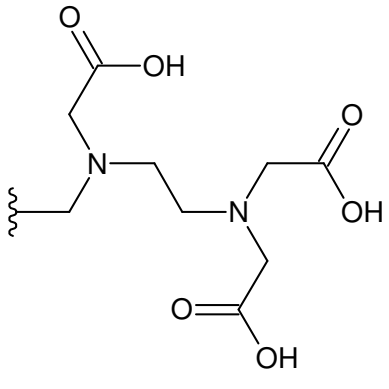

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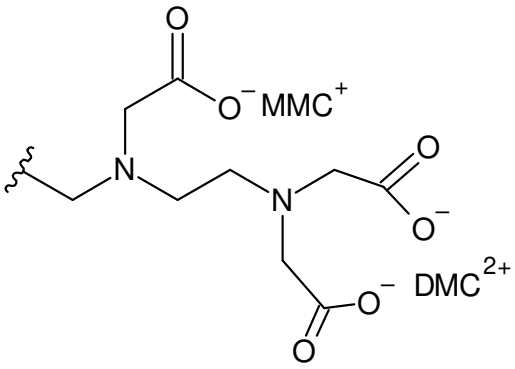
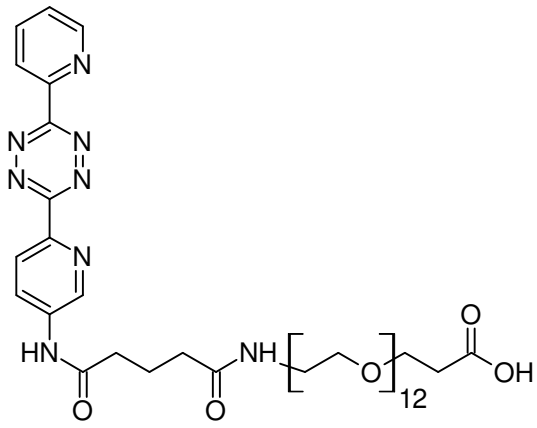


.

In relation to Formula (1), y is an integer in a range of from 1 to 13; preferably y is an integer in a range of from 1 to 11; more preferably y is an integer in a range of from 2 to 9; more preferably y is an integer in a range of from 2 to 7; more preferably y is an integer in a range of from 2 to 6; more preferably y is an integer in a range of from 2 to 4. Most preferably, y is 3.

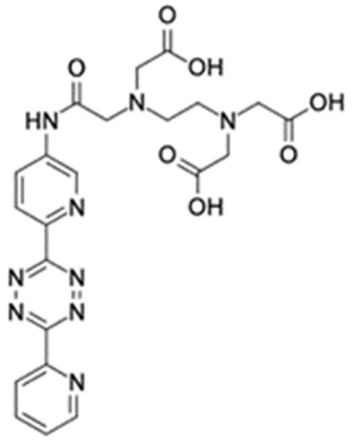
Preferably, the compounds of Formula (1) do not comprise ^{18}F, more preferably the compounds of Formula (1) do not comprise a radionuclide.

Preferably, the compound of Formula (1) is not



or a salt, solvate, or hydrate thereof.

Preferably, the compound of Formula (1) is selected from the group consisting of:



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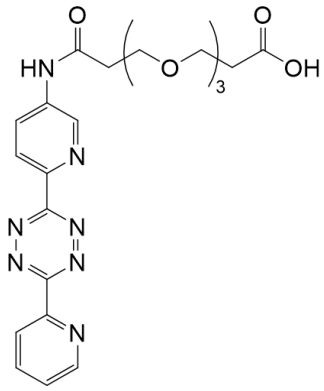

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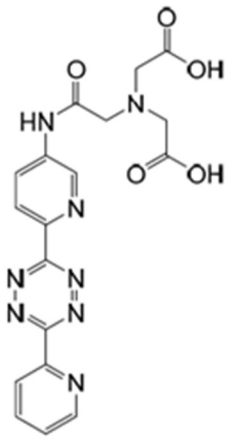

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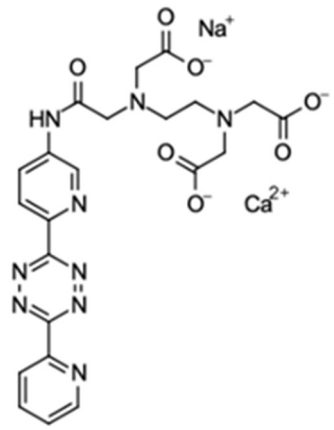

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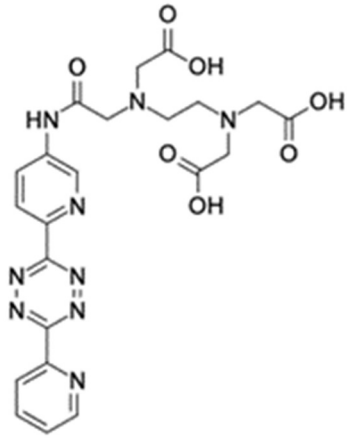
More preferably, the compound of Formula (1) is:



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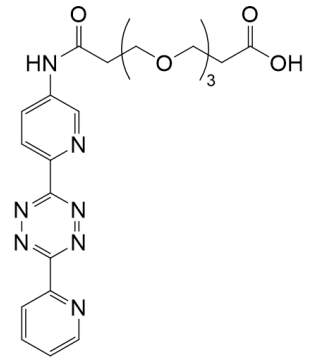
More preferably, the compound of Formula (1) is:



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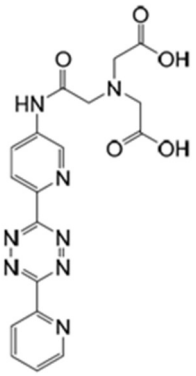
More preferably, the compound of Formula (1) is:



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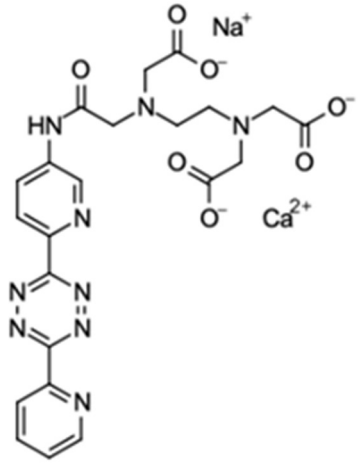
Most preferably, the compound of Formula (1) is:



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Compositions of the disclosure

The disclosure also pertains to a composition comprising a compound according to Formula (1), or the salt, hydrate, or solvate thereof. Preferably, the composition is a pharmaceutical composition. Preferably, the composition of the disclosure further comprises a pharmaceutically acceptable carrier. It is also preferred that if a salt of a compound of Formula (1) is included in the composition of the disclosure, a pharmaceutically acceptable salt is used.

Combinations of the disclosure

The disclosure also relates to a combination of (A1) a compound according to Formula (1), or the salt, hydrate, or solvate thereof; and/or (A2) a composition according to the disclosure; with (B) a dienophile or a salt, solvate, or hydrate thereof.

Preferably, the combination of the disclosure is a kit. More preferably, the combination of the disclosure is a kit wherein (A1) and/or (A2) is/are physically separated from (B).

Preferably, a dienophile as used herein comprises an eight-membered non-aromatic cyclic mono-alkenylene moiety comprising at least one allylic carbon, and optionally comprising one or more heteroatoms, preferably the heteroatom is N, O, or Si. The eight-membered non-aromatic cyclic mono-alkenylene moiety is optionally substituted. Preferably, the eight-membered non-aromatic cyclic mono-alkenylene moiety is a cyclooctene moiety, more preferably a *trans*-cyclooctene (TCO) moiety. Most preferably, the *trans*-cyclooctene moiety is an all-carbon ring.

Preferably, at least five, more preferably at least six, and most preferably at least seven, members of the eight-membered non-aromatic cyclic mono-alkenylene moiety are not substituted. Preferably, the vinylic carbons are not substituted, *i.e.* are CH. As such, if the dienophile comprises a *trans*-cyclooctene moiety that is an all-carbon ring, then the *trans*-cyclooctene moiety preferably is a ring with one double bond between two CH moieties, *i.e.* -CH=CH-, and the ring further comprises at least three, more preferably at least four, and most preferably at least five CH_2 moieties. The substituted members are preferably N or C, more preferably C.

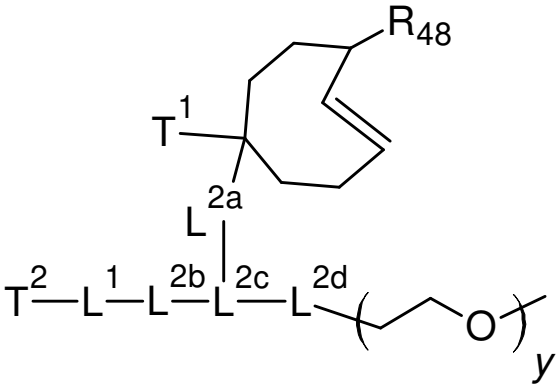
Preferably, the dienophile comprises a payload that is released upon reacting with a tetrazine of Formula (1). The payload is preferably a drug or a chelating moiety comprising a a radionuclide for imaging. If the dienophile comprises a payload, then at least one allylic carbon of the eight-membered non-aromatic cyclic mono-alkenylene moiety is directly linked to a cleavable bond. The cleavable bond comprises at least one S, N, NH, or O, and is selected from the group consisting of carbamate, thiocarbamate, carbonate, thiocarbonate, ether, ester, thioether, and thioester bonds. More preferably, the cleavable bond is selected from the group consisting of carbamate, ether, and ester bonds. Most preferably, the cleavable bond is a carbamate. The at least one S, N, NH, or O of the cleavable bond is part of the payload, or part of an optional spacer between the cleavable bond and the payload. Preferably, the optional spacer is a self-immolative linker. Self-immolative linkers are well-known in the art.

Preferably, the dienophile in relation to the disclosure is as described in WO 2020/256546, which is incorporated herein by reference, in particular in accordance with any one of claims 1 to 4 thereof, or any embodiment thereof, more preferably as described at page 44, line 5, to page 74, last line, or any embodiment thereof.

Without wishing to be bound by theory, it is believed that optionally present other substituents on the eight-membered non-aromatic cyclic mono-alkenylene moiety do not qualitatively influence the release of the payload upon reaction with a diene. In other words, the payload will be released upon reaction with a diene, regardless of whether other substituents are present on the eight-membered non-aromatic cyclic mono-alkenylene moiety. Several mechanisms for the release of the payload are known in the art. These are for example described in WO 2020/256546, in particular in Scheme 2 on page 39.

Dienophiles can be synthesized by the skilled person on the basis of known synthesis routes to cyclooctenes and corresponding hetero atom(s)-containing rings. The skilled person further is aware of the wealth of cyclooctene derivatives that can be synthesized via the ring closing metathesis reaction using Grubbs catalysts. As mentioned above, the TCO possibly includes one or more heteroatoms in the ring. This is as such sufficiently accessible to the skilled person [e.g. WO2016025480]. Reference is made, e.g., to the presence of a thioether in TCO: [Cere et al. J. Org. Chem. 1980, 45, 261]. Also, e.g., an - O-SiR_2-O moiety in TCO: [Prevost et al. J. Am. Chem. Soc. 2009, 131, 14182]. References to TCO syntheses wherein the allylic positioned leaving group (R_{48}) is an ether, ester, carbonate, carbamate or a thiocarbamate are: [Versteegen et al Angew. Chem. Int. Ed. 2018, 57, 10494], and [Steiger et al Chem Comm 2017, 53, 1378].

Preferably, the dienophile is a compound or a salt, hydrate, or solvate thereof; wherein said compound has a structure according to Formula (2):



Formula (2); wherein

L^1 is selected from the group consisting of linear or branched C_4-C_{12} alkylene, C_3-C_8 (hetero)cycloalkylene, C_6-C_{12} arylene, and C_4-C_{11} heteroarylene; preferably L^1 is linear or

branched C_4-C_{12} alkylene, more preferably L^1 is linear or branched C_4-C_{10} alkylene, and most preferably L^1 is linear C_5-C_6 alkylene;

L^{2a}, L^{2b}, and L^{2d} are each independently selected from the group consisting of -$C(O)NL^{2T}$-, -$NL^{2T}C(O)$-, -O-, -S-, -NL^{2T}-, -N=N-, and -C(O)-; wherein L^{2T} is hydrogen or methyl, preferably L^{2T} is hydrogen;

L^{2c} is selected from the group consisting of C_1-C_8 (hetero)alkanetriyl, C_5-C_6 (hetero)arenetriyl, C_3-C_7 cycloalkanetriyl, and C_2-C_7 heterocycloalkanetriyl; preferably L^{2c} is C_1-C_8 (hetero)alkanetriyl, more preferably L^{2c} is C_1-C_8 alkanetriyl, and most preferably L^{2c} is C_4-C_6 alkanetriyl;

T^1 is selected from the group consisting of -OT^{1A}, hydrogen, C_2-C_6 alkyl, C_6 aryl, C_4-C_5 heteroaryl, C_3-C_6 cycloalkyl, C_5-C_{12} alkyl(hetero)aryl, C_5-C_{12} (hetero)arylalkyl, C_4-C_{12} alkylcycloalkyl, -$N(T^{1A})_2$, -ST^{1A}, -SO_3H, -$C(O)T^{1A}$, -$C(O)OT^{1A}$, -O-$C(O)T^{1A}$ -$C(O)N(T^{1A})_2$, -$N(T^{1A})_2$-CO-T^{1A}, and -$Si(T^{1A})_3$; preferably T^1 is -OT^{1A}; and most preferably T^1 is -OH; each T^{1A} is independently selected from the group consisting of hydrogen, (hetero)alkyl, (hetero)alkenyl, (hetero)alkynyl, (hetero)aryl, and an amino acid residue; preferably T^{1A} is hydrogen or methyl, more preferably T^{1A} is hydrogen;

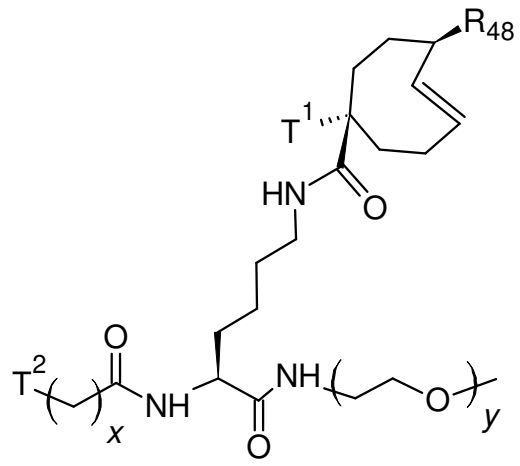
T^2 is a bioconjugation moiety or a group -L^3-C^B; preferably the bioconjugation moiety is N-maleimidyl; L^3 is a residue of a bioconjugation moiety; preferably L^3 is a residue of a maleimidyl moiety or a residue of an N-hydroxysuccinimidyl moiety;

C^B is selected from the group consisting of proteins, nucleic acids, peptides, carbohydrates, aptamers, lipids, small organic molecules, polymers, LNA, PNA, amino acids, peptoids, chelating moieties, fluorescent dyes, phosphorescent dyes, organic particles, gels, cells, and combinations thereof; preferably C^B is a protein, more preferably C^B is an antibody or a diabody, even more preferably C^B is a diabody, and most preferably C^B is AVP0458 consisting of two monomers, wherein each of the two monomers has an amino acid sequence according to SEQ ID NO: 1;

y is an integer in a range of from 1 to 50; preferably y is an integer in a range of from 10 to 40, more preferably in a range of from 12 to 37, even more preferably in a range of from 15 to 35, more preferably still in a range of from 20 to 30, and most preferably in a range of from 23 to 25; and

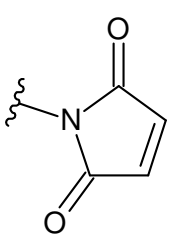
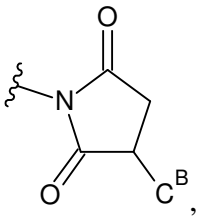
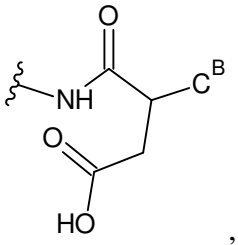
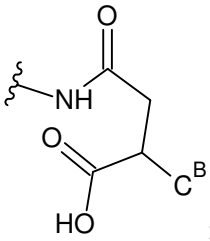
R_{48} is a releasable group, preferably R^{48} is -O-CO-C^A; wherein C^A is a payload, preferably C^A is a drug; preferably the drug is linked to the moiety -O-CO- via a secondary or tertiary nitrogen atom that is part of the drug, forming a carbamate; preferably the drug is monomethyl auristatin E (MMAE).

More preferably, the compound according to Formula (2) is a compound is according to Formula (3):

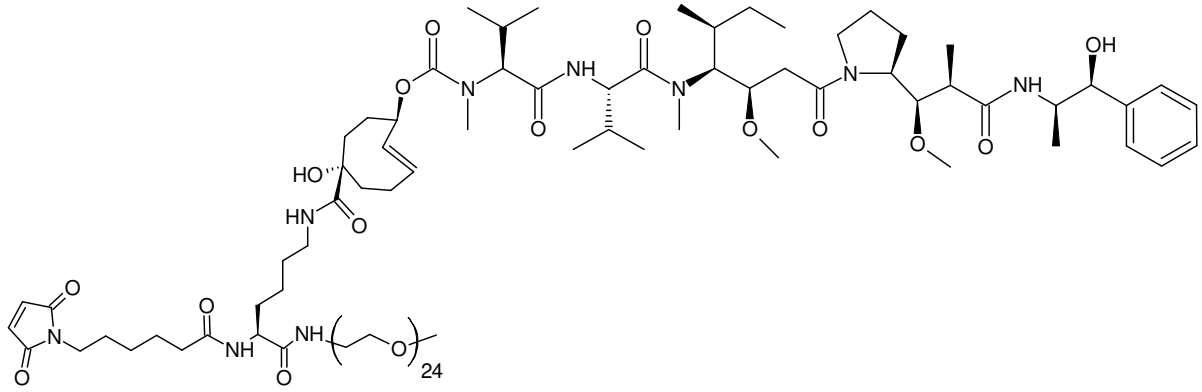


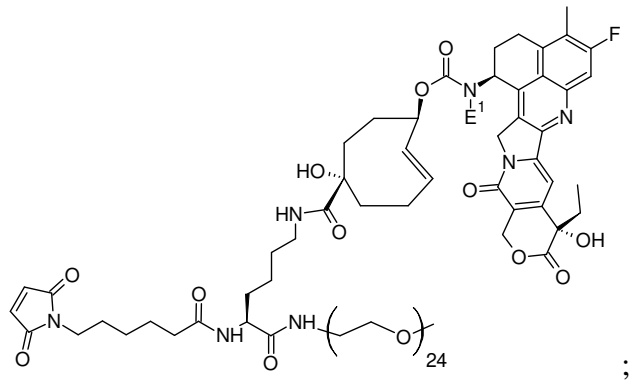
Formula (3); wherein x is an integer in a range of from 4 to 12; preferably x is an integer in a range of from 4 to 8, more preferably x is an integer in a range of from 4 to 6.

In Formula (2) and Formula (3), it is preferred that T^2 is selected from the group consisting of

 ,  ,  , and  ; wherein C^B is a protein.

Even more preferably, the dienophile is:

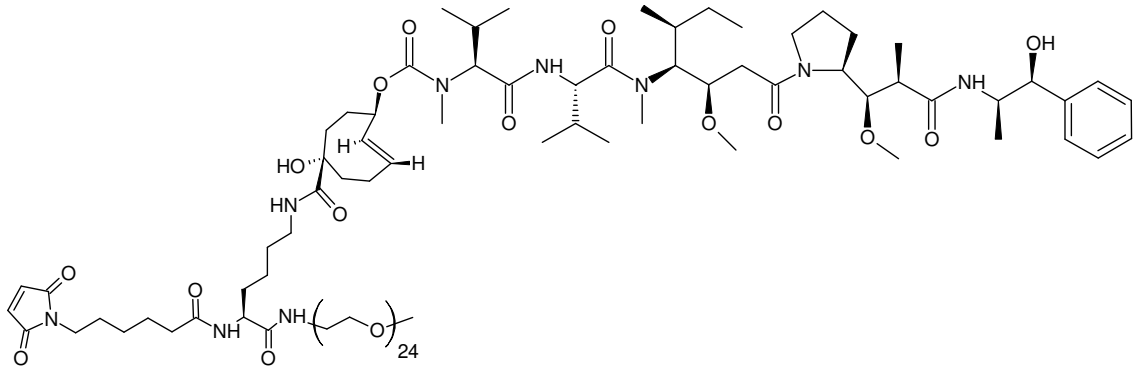




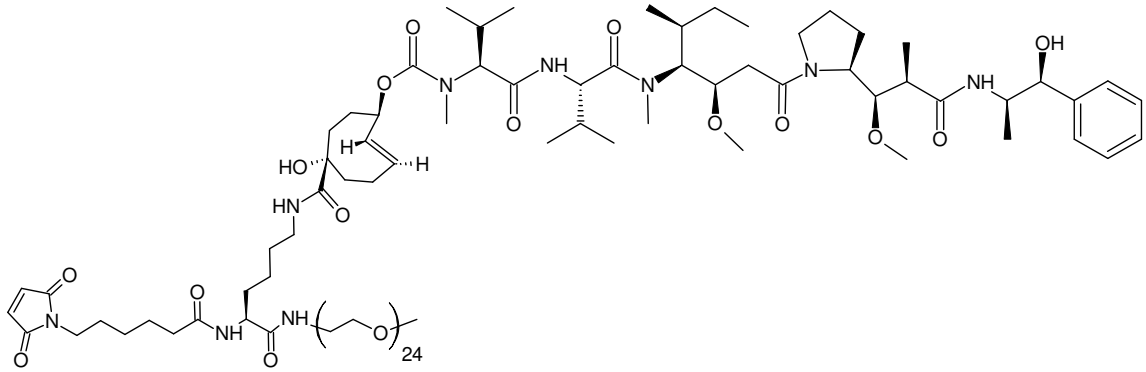
; or ; or a salt, hydrate, or solvate thereof.

As used herein in relation to dienophiles of the disclosure **E¹** is -H or -CH_3.

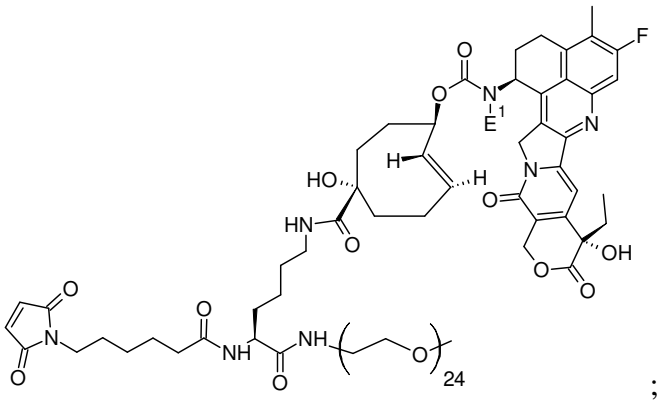
More preferably still, the dienophile is:



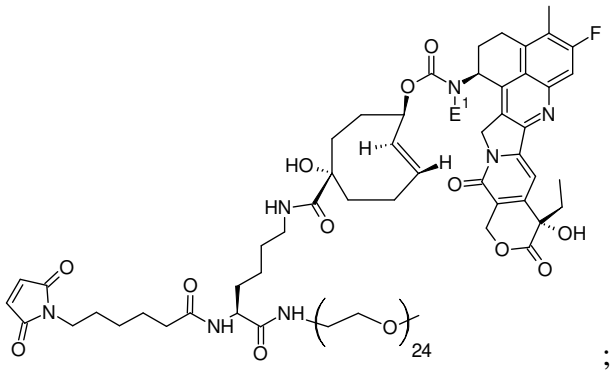
; or



; or

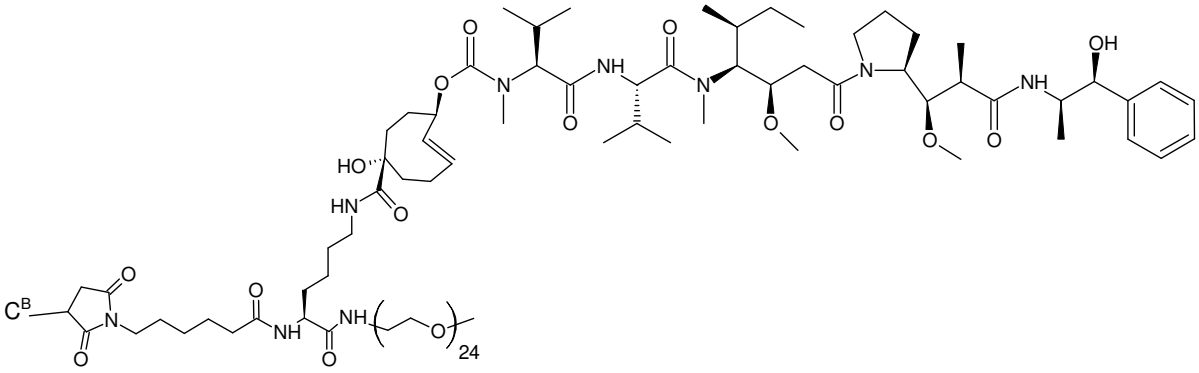


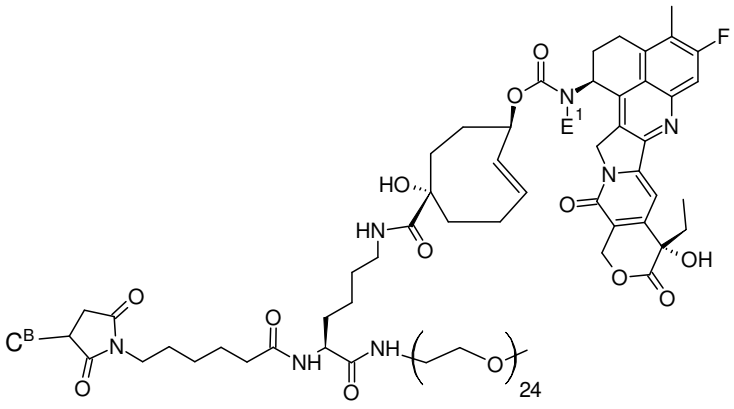
; or



; or a salt, hydrate, or solvate thereof.

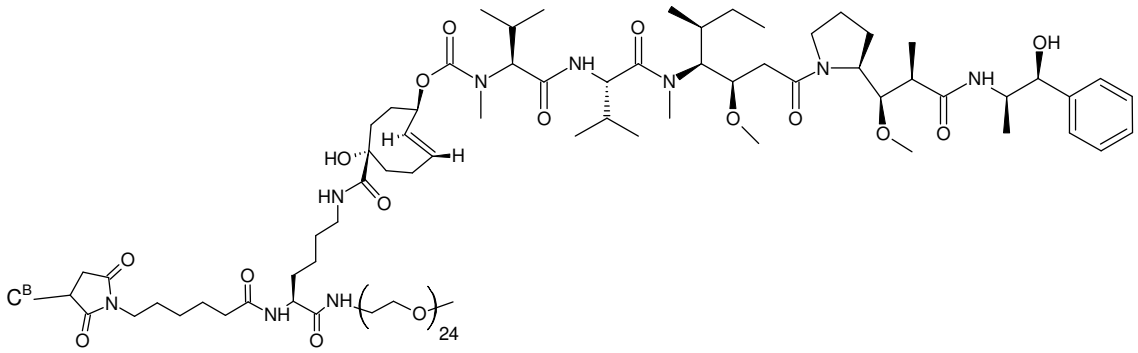
Yet more preferably, the dienophile is:



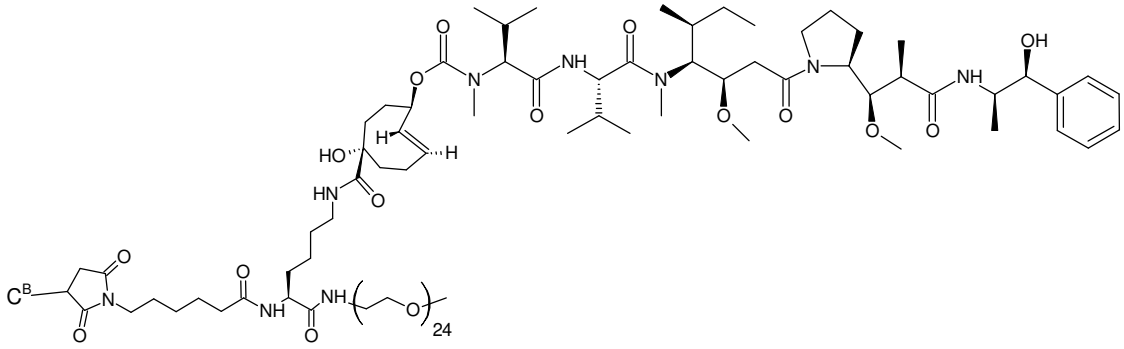


; or ; wherein C^B is AVP0458 consisting of two monomers, wherein each of the two monomers has an amino acid sequence according to SEQ ID NO: 1; preferably C^B is linked to the maleimidyl group via a sulfur atom that is part of C^B, preferably the sulfur atom is part of a cysteine.

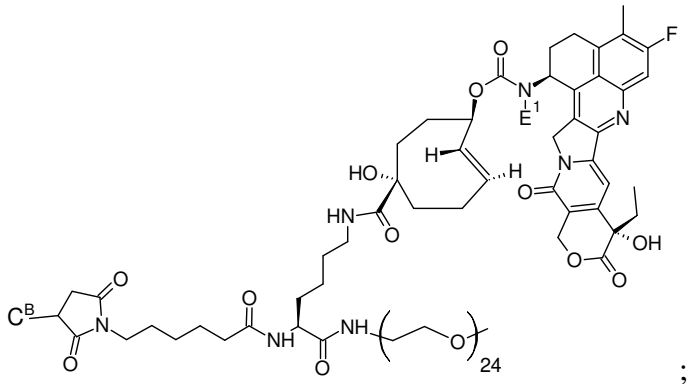
Most preferably, the dienophile is:



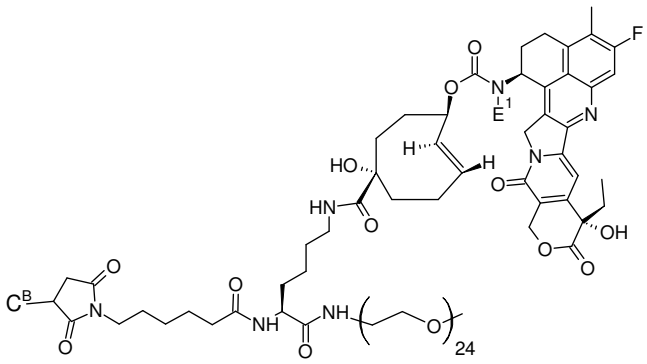
; or



; or



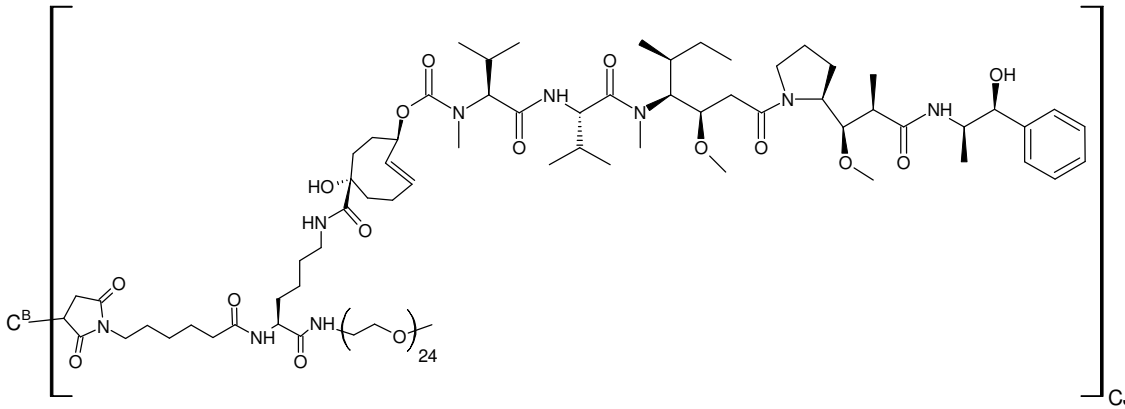
; or



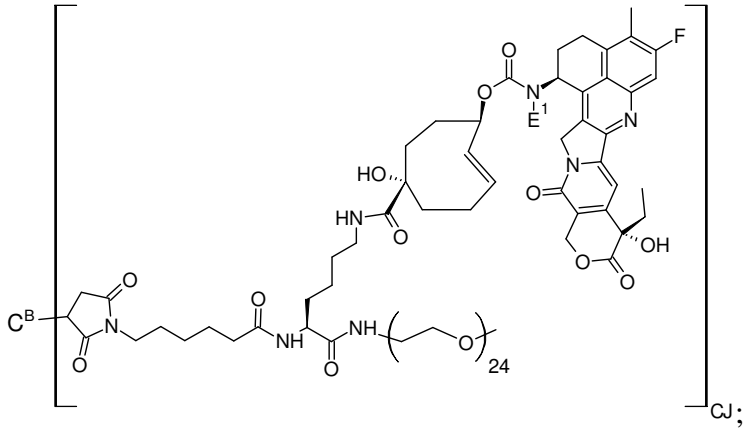
; or a salt, hydrate, or solvate therof;

wherein C^B is AVP0458 consisting of two monomers, wherein each of the two monomers has an amino acid sequence according to SEQ ID NO: 1; preferably C^B is linked to the maleimidyl group via a sulfur atom that is part of C^B, preferably the sulfur atom is part of a cysteine.

In other preferred embodiments, the dienophile is a conjugate, or a salt, hydrate, or solvate thereof. Preferably, the conjugate is:



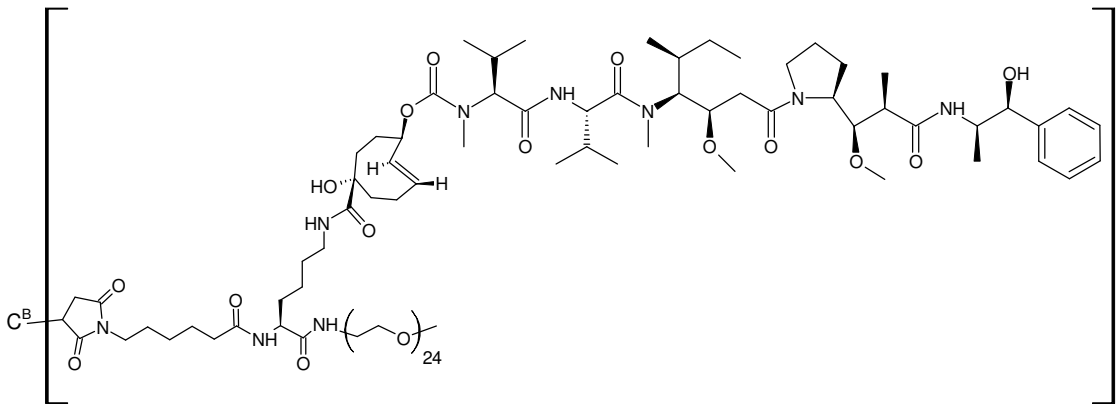
; or



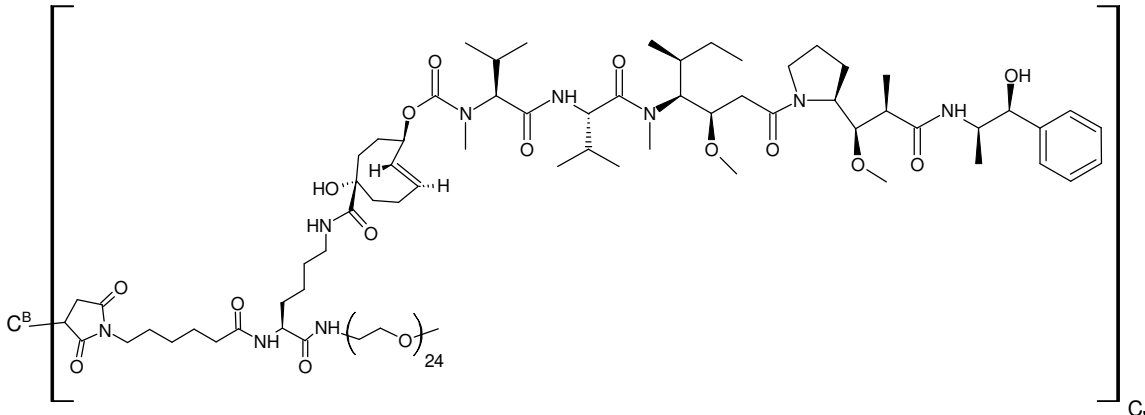
;

wherein CJ is in a range of from 1 to 12; wherein C^B is AVP0458 consisting of two monomers, wherein each of the two monomers has an amino acid sequence according to SEQ ID NO: 1; preferably CJ is of from 2 to 10, more preferably of from 2.5 to 8, even more preferably of from 3 to 6, even more preferably still of from 3.5 to 4, and most preferably about 4; preferably C^B is linked to each maleimidyl group via a sulfur atom, preferably the sulfur atom is part of a cysteine.

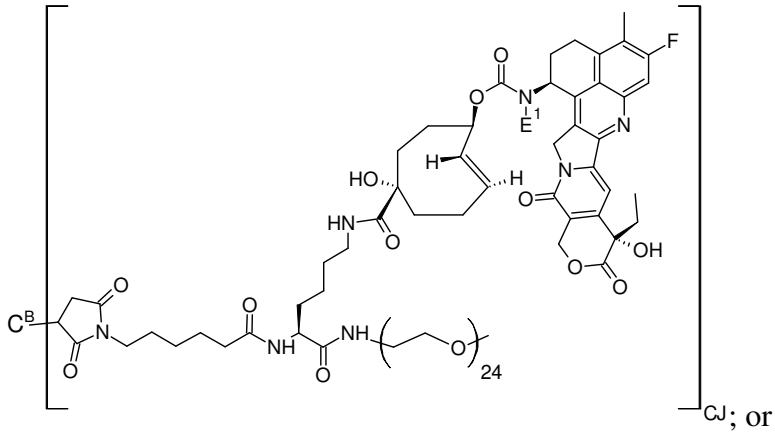
More preferably, the conjugate is:



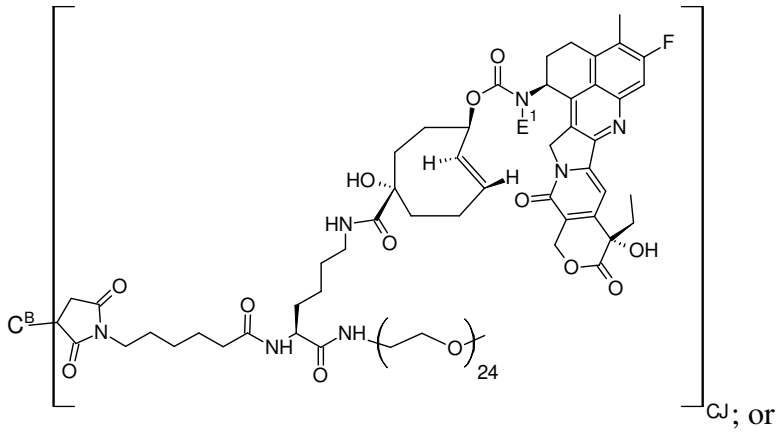
; or



; or



; or



; or a salt, hydrate, or solvate thereof; wherein CJ is in a range of from 1 to 12; wherein C^B is AVP0458 consisting of two monomers, wherein each of the two monomers has an amino acid sequence according to SEQ ID NO: 1; preferably CJ is of from 2 to 10, more preferably of from 2.5 to 8, even more preferably of from 3 to 6, even more preferably still of from 3.5 to 4, and most preferably about 4; preferably C^B is linked to each maleimidyl group via a sulfur atom, preferably the sulfur atom is part of a cysteine.

AVP0458

As used herein, AVP0458 refers to a TAG72-binding diabody derived from the CC49 antibody. AVP0458 is a diabody consisting of two monomers, each monomer having an amino acid sequence according to SEQ ID NO:1:

SEQ ID NO:1 (amino acid sequence of AVP0458 diabody monomer):
SVQLQQSDAELVKPGASVKISCKASGYTFTDHAIHWVKQNPEQGLEWIGYFSPGNDD
FKYNERFKGKATLTADKSSSTAYLQLNSLTSEDSAVYFCTRSLNMAYWGQGTSVTV
SSGGGGSDIVMTQS**C**SS**C**PVSVGEKVTLSCKSSQSLLYSGNQKNYLAWYQQKPGQSP
KLLIYWASTRESGVPDRFTGSGSGTDFTLSISSVETEDLAVYYCQQYYSYPLTFGAGT
KLVLKR

Herein, the underlining indicates the cysteines that are preferably modified with or linked to a dienophile of the disclosure or the remainder thereof if AVP0458 is itself part of the dienophile of the disclosure.

Thus, in SEQ ID NO: 1 it is preferred that at least one of the underligned cysteines, more preferably both underlined cysteines, is modified with or linked to a dienophile according to the disclosure. In other words: it is preferred that the sulfur atom of the underlined cysteines is coupled to a moiety T^2 as defined herein, preferably T^2 is the residue of an N-maleimidyl group.

Non-therapeutic methods using and uses for using compounds of Formula (1)

In some embodiments, the disclosure pertains to non-therapeutic methods and non-therapeutic uses. Preferably, the dienophile used therein is as described in relation to the combination of the disclosure.

For the non-therapeutic method of the disclosure it is preferred that the compound of Formula (1) (viz. (ia)), and/or the composition of the disclosure (viz. (iia)), and the dienophile are further contacted with a solvent. The skilled person is aware of suitable solvents for a reaction between a tetrazine and a dienophile. Preferably, the solvent comprises water, and more preferably the solvent is water.

For the non-therapeutic use, the click reaction is preferably a bioorthogonal click reaction. Preferably, the click reaction is performed *in vitro*, although non-therapeutic reactions *in vivo* can be carried out as well.

Methods for preparing compounds of Formula (1)

The disclosure also relates to a method for preparing preferred compounds of Formula (1). In step (a) thereof, SM1a or SM1b is reacted with a reagent selected from the group consisting of SM2, SM3, and SM4. Preferably, SM1a is used in step (a).

If SM1a is used in step (a), then step (b) is carried out after step (a). In step (b), the reaction product of step (a) is subjected to oxidation. Preferably, in step (b) the oxidation is performed by adding sodium nitrate, optionally in the presence of an acid, preferably formic acid. If SM1b is used in step (a), then step (b) does not need to be carried out.

In the entirely optional step (c) the reaction product of step (a) or step (b) is subjected to salt formation. The skilled person is aware of standard procedures to form salts from compounds comprising one or more carboxylic acid groups. Preferably, sodium and/or calcium salts are formed in step (c).

For all of steps (a), (b), and (c), it is preferred that the reagents are contacted with a solvent. The skilled person is aware of suitable solvents to be used in said steps.

The present disclosure is herein described with respect to particular embodiments, but the disclosure is not limited thereto but only by the claims. 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.

The verb "to comprise", and its conjugations, as used in this description and in the claims is used in its non-limiting sense to mean that items following the word are included, but items not specifically mentioned are not excluded.

In addition, reference to an element by the indefinite article "a" or "an" does not exclude the possibility that more than one of the element is present, unless the context clearly requires that there is one and only one of the elements. The indefinite article "a" or "an" thus usually means "at least one".

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 disclosure, the only relevant components of the device are A and B.

The compounds disclosed herein may occur in different tautomeric forms. The compounds disclosed herein are meant to include all tautomeric forms, unless stated otherwise. When the structure of a compound is depicted as a specific tautomer, it is to be understood that the disclosure of the present application is not limited to that specific tautomer, unless stated otherwise.

Unless stated otherwise, the compounds disclosed herein and/or groups thereof may be protonated or deprotonated. It will be understood that it is possible that a compound may bear multiple charges which may be of opposite sign. For example, in a compound containing an amine and a carboxylic acid, the amine may be protonated while simultaneously the carboxylic acid is deprotonated.

In several formulae, groups or substituents are indicated with reference to letters such as “A”, “B”, “X”, “Y”, and various (numbered) “R” groups. In addition, the number of repeating units may be referred to with a letter, e.g. n in $-(CH_2)_n-$. 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.

Herein, reference is made to "alkyl", and the like. The number of carbon atoms that these groups have, excluding the carbon atoms comprised in any optional substituents as defined below, can be indicated by a designation preceding such terms (e.g. “C_1-C_8 alkyl” means that said alkyl may have from 1 to 8 carbon atoms). For the avoidance of doubt, a butyl group substituted with a $-OCH_3$ group is designated as a C_4 alkyl, because the carbon atom in the substituent is not included in the carbon count.

As used herein, alkyl groups are unsubstituted and have the general formula C_nH_{2n+1} and may be linear or branched. Examples of alkyl groups include methyl, ethyl, propyl, 2-propyl, t-butyl, 1-hexyl, 1-dodecyl, etc. Thus, C_{1-3} alkyl groups are methyl, ethyl, 1-propyl, and 2-propyl.

The term "salt thereof” means a compound formed when an acidic proton, typically a proton of an acid, is replaced by a cation, such as a metal cation or an organic cation and the like. The term "salt thereof” also means a compound formed when an amine is protonated. Where applicable, the salt is a pharmaceutically acceptable salt, although this is not required for salts that are not intended for administration to a patient. For example, in a salt of a compound the compound may be protonated by an inorganic or organic acid to form a cation, with the conjugate base of the inorganic or organic acid as the anionic component of the salt.

The term "pharmaceutically accepted salt” means a salt that is acceptable for administration to a patient, such as a mammal (salts with counter-ions having acceptable mammalian safety for a given dosage regime). Such salts may be derived from pharmaceutically acceptable inorganic or organic bases and from pharmaceutically acceptable inorganic or organic acids.

"Pharmaceutically acceptable salt" refers to pharmaceutically acceptable salts of a compound, which salts are derived from a variety of organic and inorganic counter ions

known in the art and include, for example, sodium, potassium, calcium, magnesium, ammonium, tetraalkylammonium, etc., and when the molecule contains a basic functionality, salts of organic or inorganic acids, such as hydrochloride, hydrobromide, formate, tartrate, besylate, mesylate, acetate, maleate, oxalate, etc.

It will be understood that herein, the terms "moiety" and "group" are used interchangeably when referring to a part of a molecule.

It will be understood that when a heteroatom is denoted as $-X(R')_2-$, wherein X is the heteroatom and R' is a certain moiety, then this denotes that two moieties R' are attached to the heteroatom.

It will be understood that when a group is denoted as, for example, $-((R_{51})_2-R_{52})_2-$ or a similar notation, in which R_{51} and R_{52} are certain moieties, then this denotes that first, it should be written as $-R_{51}-R_{51}-R_{52}-R_{51}-R_{51}-R_{52}-$ before the individual R_{51} and R_{52} moieties are selected, rather than first selecting moieties R_{51} and R_{52} and then writing out the formula.

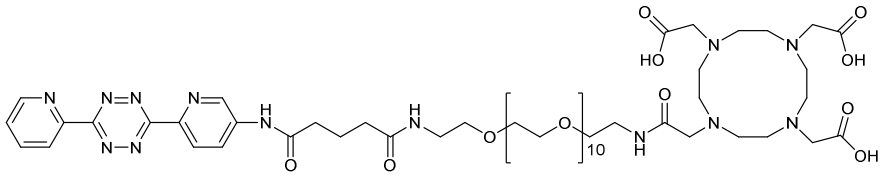
Examples

Example 1: General methods

All reagents, chemicals, materials and solvents were obtained from commercial sources and were used as received, including nitrile starting compounds that have not been described. All solvents were of AR quality. Analytical thin layer chromatography (TLC) was performed on Kieselgel F-254 precoated silica plates. Column chromatography was carried out on Screening Devices B.V. silica gel (flash: 40-63 μm mesh; normal: 60-200 μm mesh). Reverse phase chromatography was performed using a Büchi Reveleris C18 column (80 g). ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Avance III HD (400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR) spectrometer or a JEOL (500 MHz for ^{1}H NMR) at 298 K. Chemical shifts are reported in ppm downfield from TMS at rt. Abbreviations used for splitting patterns are s = singlet, d = doublet, dd = double doublet, t = triplet, q = quartet, m = multiplet and br = broad. HPLC-PDA/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). HPLC-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 and typically a gradient (5 % to 100 % in 10 min, held at 100 % for a further 3 min) of acetonitrile (MeCN) in H_2O (both containing 0.1 % formic acid) at 298 K.

Example 2: Synthesis of tetrazines

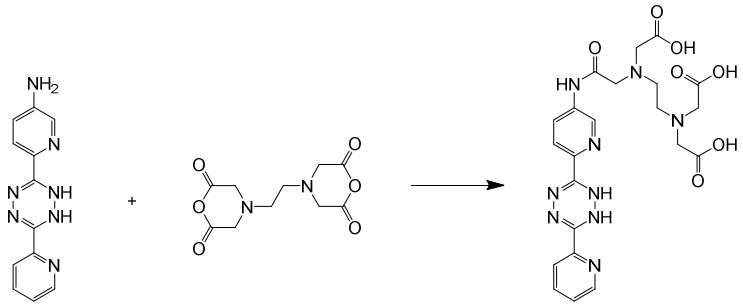
*Example 2.1: Reference compound **2.1***



2.1

The synthesis of 2,2',2''-(10-(2,40,44-Trioxo-44-((6-(6-(pyridine-2-yl)-1,2,4,5-tetrazin-3-yl)pyridine-3-yl)amino)-6,9,12,15,18,21,24,27,30,33,36-undecaoxa-3,39-diazatetratetracontyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid (**2.1**) was reported in Rossin et al., Angew. Chem. Int. Ed. 2010, 49, 3375 –3378.

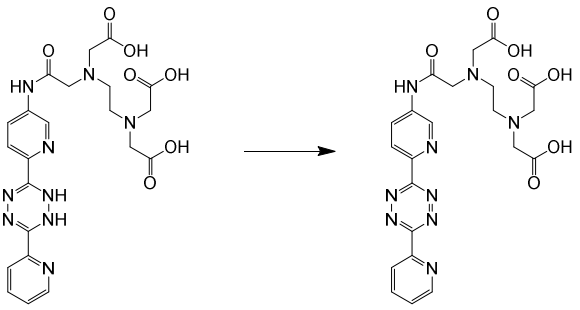
*Example 2.2: 2,2'-((2-((Carboxymethyl)(2-oxo-2-((6-(6-(pyridin-2-yl)-1,2-dihydro-1,2,4,5-tetrazin-3-yl)pyridin-3-yl)amino)ethyl)amino)ethyl)azanediyl)diacetic acid (**2.3**)*



2.2 **2.3**

Compound **2.2** was prepared according to Blackman et al. J. Am. Chem. Soc. 2008, 130, 13518-13519. Ethylenediaminetetraacetic dianhydride (1.86 g, 7.26 mmol) was dissolved in dry DMSO (3 mL) by gentle heating. The mixture was allowed to cool to room temperature and a solution of **2.2** (450 mg, 1.78 mmol) in DMSO (11 mL) was slowly added. The orange, hazy mixture was stirred at room temperature under an atmosphere of argon for 5 h. Subsequently water (0.1 mL) was added and the mixture was stirred for 30 min. **2.3** mixture was used without further purification. ESI-MS: m/z Calc. for $C_{22}H_{25}N_9O_7$ 527.19 Da; Obs. $[M+H]^+$ 528.42 Da and $[M-H^+]^-$ 526.50 Da.

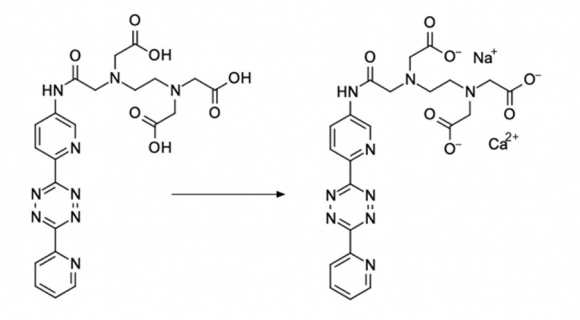
*Example 2.3: 2,2'-((2-((Carboxymethyl)(2-oxo-2-((6-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-yl)amino)ethyl)amino)ethyl)azanediyl)diacetic acid (**2.4**)*



2.3 **2.4**

The crude reaction mixture of **2.3** was diluted with water (15 mL) and acidified by addition of formic acid (0.2 mL). Sodium nitrite (400 mg, 5.79 mmol) was added and the pink mixture was stirred in a closed flask at room temperature for 1 h. An aqueous solution of 1 M ammonium acetate (30 mL) was added and the pink suspension was centrifuged at 3000 rpm for 10 min. The clear, dark pink supernatant was isolated and purified by reversed-phase chromatography (C18 column, gradient of 5% MeCN / 0.1 M aqueous ammonium acetate to 25%). The combined product fractions were freeze dried, redissolved in water (25 mL), and again freeze dried and redissolved in water. To the pink solution was added formic acid (0.25 mL), which caused the product to precipitate. The suspension was centrifuged at 3000 rpm for 10 min, after which the clear, faint pink supernatant was discarded. The pink solid was washed with water (25 mL) and centrifuged, for two more times, and then washed with MeCN and centrifuged, for two more times. The remaining pink solid was dried in vacuo to give 486 mg (52% overall yield) of **2.4**. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.41 (br.s, 3H), 10.78 (s, 1H), 9.13 (d, J = 2.5 Hz, 1H), 8.94 (dd, J = 4.8, 1.7 Hz, 1H), 8.62 (m, 2H), 8.52 (dd, J = 8.7, 2.5 Hz, 1H), 8.16 (td, J = 7.8, 1.8 Hz, 1H), 7.73 (dd, J = 7.8, 4.7 Hz, 1H), 3.53 (m, J = 10.4 Hz, 8H), 2.85 (s, 4H) ppm. ^{13}C NMR (101 MHz, DMSO-d6): δ 173.40, 172.97, 171.94, 163.52, 163.27, 151.08, 150.67, 144.47, 142.13, 138.49, 138.28, 127.05, 126.75, 125.28, 124.66, 58.55, 55.68, 55.37, 52.58, 52.22 ppm. ESI-MS: m/z Calc. for $C_{22}H_{23}N_9O_7$ 525.17 Da; Obs. $[M+H]^+$ 526.33 Da and $[M-H]^-$ 524.42 Da.

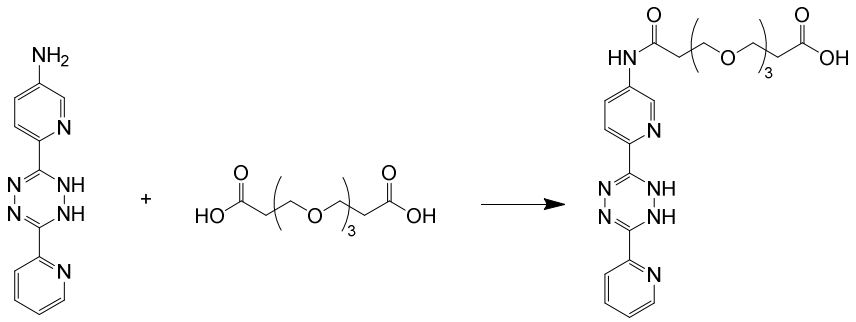
*<u>Example 2.4:</u> Calcium sodium 2-({2-[bis(carboxylatomethyl)amino]ethyl}[({6-[6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl]pyridin-3-yl}carbamoyl)methyl]amino)acetate (**2.5**)*



2.4 **2.5**

1M Sodium acetate was prepared by dissolving sodium acetate trihydrate in milliQ-H_2O (pH ca. 9.0) followed by acidification to pH=6.4 acidic with AcOH glacial. To an acidic suspension of **2.4** (172 mg, 0.33 mmol; 10 mg/mL) in milliQ-H_2O was added dropwise $CaCO_3$ (1.67 mL, 0.53 mmol of a 32 mg/mL homogenous suspension). Upon increasing pH, the tetrazine dissolved at pH=5.8 and $CaCO_3$ addition was halted at pH=6.5. Subsequently, 1.0 M sodium acetate (pH=6.4) was added to the tetrazine to obtain a final 0.1 M sodium acetate concentration. The solution was applied to a Sep-Pak column (10 gr, Waters) for purification (i.e. removal of excess calcium and sodium acetate components). The tetrazine remained at the top of the column and was rinsed once with 0.1 M sodium acetate followed by rinsing with milliQ-H_2O (6 volumes) prior to elution with milliQ-H_2O:MeOH (1:1) aided by vacuum pull. Tetrazine containing fractions were combined, reduced 80% by volume in vacuo, diluted with milliQ-H_2O, and freeze-dried after micropore filtration. The freeze-dried residue was redissolved in milliQ-H_2O at 50 mg/mL, micropore filtered once more, and freeze-dried to obtain **2.5** as a homogenous pink fluffy powder. ^{1}H NMR (400 MHz, D_2O) δ 8.89 (dd, *J* = 2.6, 0.6 Hz, 1H), 8.79 (ddd, *J* = 4.8, 1.7, 0.9 Hz, 1H), 8.63 – 8.55 (m, 2H), 8.37 (dd, *J*= 8.7, 2.6 Hz, 1H), 8.16 (td, *J* = 7.8, 1.7 Hz, 1H), 7.74 (ddd, *J* = 7.7, 4.7, 1.1 Hz, 1H), 3.58 (s, 2H), 3.32 – 3.06 (m, 6H), 2.77 – 2.50 (m, 4H) ppm. ^{13}C NMR (100 MHz, D_2O) δ 179.9, 179.3, 174.1, 162.9, 162.5, 150.3, 148.3, 143.9, 142.0, 139.0, 137.3, 128.9, 127.6, 125.3, 124.7, 60.5, 59.8, 54.8 ppm. ESI-MS cal. for $C_{22}H_{23}N_9O_7$ 525.17 (excl. sodium calcium), found. M+H^+ 526.25. Elemental anal. calcd for $C_{22}H_{20}CaN_9NaO_7$: Composition: C (45.1%), Ca (6.8%), N (21.5%), Na (3.9%). Measured: C (44.0%), Ca (7.2%), N (20.5%), Na (3.1%).

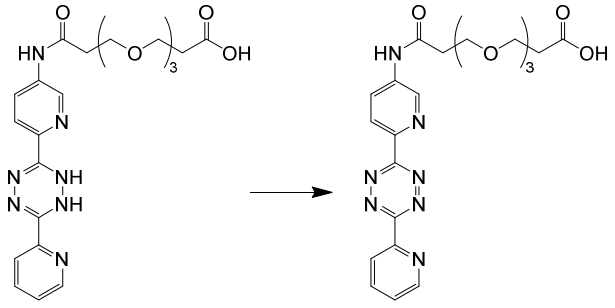
*<u>Example 2.5:</u> 3-(2-(2-(3-Oxo-3-((6-(6-(pyridin-2-yl)-1,2-dihydro-1,2,4,5-tetrazin-3-yl)pyridin-3-yl)amino)propoxy)ethoxy)ethoxy)propanoic acid (**2.6**)*



2.2 **2.6**

3,3'-((Oxybis(ethane-2,1-diyl))bis(oxy))dipropionic acid (5.90 g, 23.6 mmol) was dissolved in chloroform (100 mL), and pyridinium *p*-toluenesulfonate (0.15 g, 0.597 mmol) and EDC HCl (1.13 g, 5.92 mmol) were added. The solution was stirred at room temperature under an atmosphere of argon for 30 min. Subsequently, compound **2.2** (1.50 g, 5.92 mmol) was added, followed by DMAP (0.36 g, 2.95 mmol). The orange solution was stirred at room temperature under an atmosphere of argon for 90 min, and then washed twice with 0.5 M aqueous citric acid (60 mL). The combined organic layers were dried with sodium sulfate, filtered, and concentrated. Crude **2.6** was used without further purification. ESI-MS: m/z Calc. for $C_{22}H_{27}N_7O_6$ 485.20 Da; Obs. $[M+H]^+$ 486.25 Da and $[M-H^+]^-$ 484.33 Da.

*<u>Example 2.6:</u> 3-(2-(2-(3-Oxo-3-((6-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-yl)amino)propoxy)ethoxy)ethoxy)propanoic acid (**2.7**)*

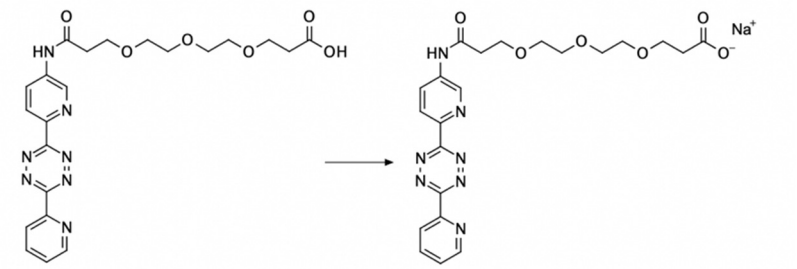


2.6 **2.7**

The crude product **2.6** was dissolved in MeCN (30 mL) and water (30 mL), and formic acid (1.5 mL) was added, followed by sodium nitrite (1.23 g, 17.8 mmol). The pink/red solution was stirred in a closed flask at room temperature for 30 min, and then diluted with water (90 mL). The mixture was filtered and purified by reversed-phase chromatography (C18 column, gradient of 15% MeCN / 0.1% aqueous formic acid to 30%). The combined product fractions were freeze-dried to give **2.7** as a pink, fluffy solid (1.32 g, 46% overall yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.61 (br.s, 1H), 8.97 (dt, J = 4.6, 1.4 Hz, 1H), 8.79 (m, J = 6.3, 2.6 Hz, 2H),

8.77 – 8.62 (m, 2H), 8.01 (td, J = 7.8, 1.8 Hz, 1H), 7.58 (ddd, J = 7.6, 4.7, 1.2 Hz, 1H), 3.85 (t, J = 5.5 Hz, 2H), 3.80 (t, J = 5.8 Hz, 2H), 3.74 (s, 4H), 3.72 – 3.59 (m, 4H), 2.76 (t, J = 5.5 Hz, 2H), 2.65 (t, J = 5.8 Hz, 2H) ppm. ^{13}C NMR (101 MHz, $CDCl_3$): δ 174.18, 171.32, 163.53, 163.02, 150.91, 150.07, 143.56, 141.93, 138.55, 137.57, 127.36, 126.55, 125.27, 124.38, 70.70, 70.48, 70.20, 67.02, 66.78, 37.91, 35.40 ppm. ESI-MS: m/z Calc. for $C_{22}H_{25}N_7O_6$ 483.19 Da; Obs. $[M+H]^+$ 484.50 Da and $[M-H]^-$ 482.33 Da.

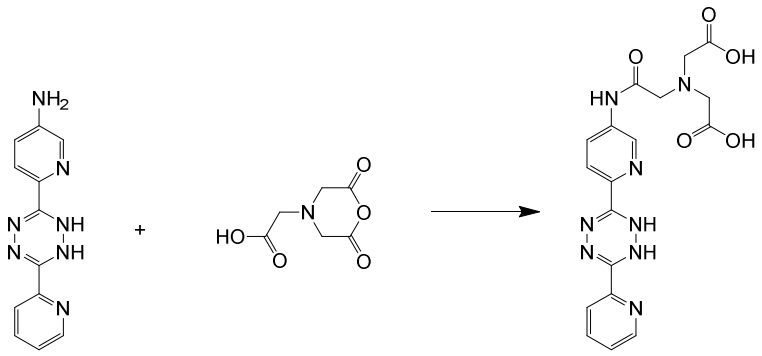
*<u>Example 2.7:</u> Sodium 3-(2-{2-[2-({6-[6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl]pyridin-3-yl}carbamoyl)ethoxy]ethoxy}ethoxy)propanoate (**2.8**)*



2.7 **2.8**

To an acidic suspension of **2.7** (5.65 mg, 10 µmol, 10 mg/mL) in milliQ-H_2O was added dropwise $NaHCO_3$ (0.13 mL, 13 µmol of a 8.4 mg/mL solution). Upon increasing pH, the tetrazine dissolved at pH=4.5 and $NaHCO_3$ addition was halted at pH=7.2. The solution was applied to a C18 Sep-Pak column for purification (i.e. removal of excess sodium). The tetrazine remained at the top of the column and was rinsed with milliQ-H_2O (6 volumes) prior to elution with milliQ-H_2O:MeOH (1:1) aided by vacuum pull. Tetrazine containing fractions were combined, reduced 50% by volume in vacuo and freeze-dried after micropore filter filtration. The freeze-dried residue was redissolved in milliQ-H_2O at 15 mg/mL and freeze-dried to obtain **2.8** as homogenous pink fluffy solid.

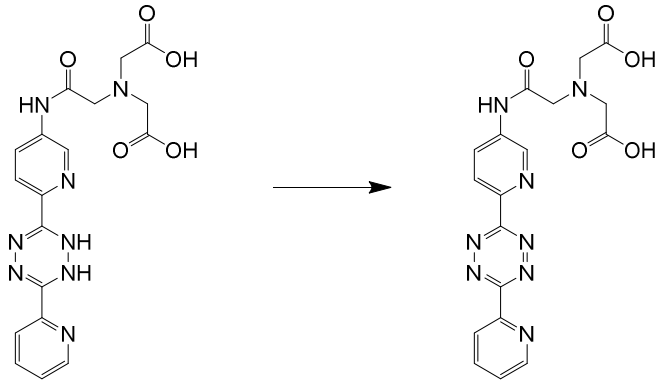
*<u>Example 2.8:</u> 2,2'-((2-Oxo-2-((6-(6-(pyridin-2-yl)-1,2-dihydro-1,2,4,5-tetrazin-3-yl)pyridin-3-yl)amino)ethyl)azanediyl)diacetic acid (**2.9**)*



2.2 **2.9**

To a solution of **2.2** (168 mg, 0.66 mmol) in DMF (5 mL) was slowly added a solution of nitrilotriacetic anhydride (114 mg, 0.66 mmol) in DMF (1 mL). The solution was stirred at room temperature under an atmosphere of argon for 20 h. Water (30 mL) and formic acid (0.3 mL) were added to precipitate the product, which was isolated by centrifugation and decantation. The precipitate was washed with MeCN (30 mL), and dried in vacuo to yield **2.9** as orange powder (217 mg, 77% yield). ^{1}H NMR (400 MHz, DMSO-d6): δ 12.63 (br.s, 2H), 10.71 (br.s, 1H), 8.95 (s, 1H), 8.88 (s, 1H), 8.84 (d, J = 2.4 Hz, 1H), 8.64 (dd, J = 4.8, 1.6 Hz, 1H), 8.19 (dd, J = 8.7, 2.5 Hz, 1H), 8.07 – 7.83 (m, 3H), 7.53 (ddd, J = 6.9, 4.8, 1.6 Hz, 1H), 3.58 (s, 4H), 3.55 (s, 2H) ppm. ESI-MS: m/z Calc. for $C_{18}H_{18}N_8O_5$ 426.14 Da; Obs. $[M+H]^+$ 427.33 Da and $[M-H]^-$ 425.42 Da.

*<u>Example 2.9:</u> 2,2'-((2-Oxo-2-((6-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-yl)amino)ethyl)azanediyl)diacetic acid (**2.10**)*



2.9 **2.10**

Compound **2.9** (120 mg, 0.282 mmol) was suspended in water (20 mL), and sodium nitrite (97 mg, 1.41 mmol) was added. The suspension was stirred at room temperature under an atmosphere of argon, and turned pink and became clear within 10 min. After 1 h, a precipitate was formed again and the mixture was stored at 4°C for 1 h. The precipitate was isolated by

centrifugation, washed with subsequently water (20 mL) and acetonitrile (25 mL), and dried in vacuo, to yield **2.10** as a pink powder (110 mg, 92%). ^{1}H NMR (400 MHz, DMSO-d6): δ 12.53 (br.s, 2H), 10.92 (br.s, 1H), 9.07 (d, J = 2.5 Hz, 1H), 8.94 (d, J = 4.2 Hz, 1H), 8.66 (d, J = 8.7 Hz, 1H), 8.60 (d, J = 7.9 Hz, 1H), 8.47 (dd, J = 8.7, 2.6 Hz, 1H), 8.16 (td, J = 7.8, 1.8 Hz, 1H), 7.73 (ddd, J = 7.7, 4.7, 1.2 Hz, 1H), 3.62 (m, 6H) ppm. ^{13}C NMR (101 MHz, DMSO-d6): δ 173.86, 172.77, 171.68, 163.55, 163.24, 151.09, 150.71, 144.69, 141.66, 138.33, 138.28, 127.08, 126.50, 125.46, 124.69, 59.58, 56.45 ppm. ESI-MS: m/z Calc. for $C_{18}H_{16}N_8O_5$ 424.12 Da; Obs. $[M+H]^+$ 425.33 Da and $[M-H]^-$ 423.42 Da.

Example 3: Inhibition of physiologically relevant target proteins by tetrazines

In this Example, the inhibition of several physiologically relevant target proteins (viz. enzymes and a transporter) by tetrazines were tested *in vitro*. The enzymes tested were cyclooxygenase (COX-1), acetyl cholinesterase (ACES), and monoamine oxidase (MAO-B), and the tested transporter was calcium channel L-type, dihydropyridine. COX-1, ACES, and MAO-B from humans was used, while calcium channel L-type, dihydropyridine from rats was used. Standard literature protocols were used, and relevant controls were included to ensure the validity of the results. The target proteins were mixed with tetrazine **2.1** (reference), **2.4**, **2.5**, **2.7**, or **2.10** (final concentration of tetrazine 10 μM) in appropriate buffers. The final concentrations for the respective target proteins depended on the standard protocol used, but for each individual target protein this concentration was the same for the different tetrazines tested. After an appropriate incubation time at an appropriate temperature, the enzymatic activity of the enzyme or the ligand binding in case of the transporter was tested in accordance with standard procedures from literature. From this remaining enzymatic activity or ligand binding, the percentage of inhibition was determined.

The results of these experiments are presented in Table 1 below. Therein, it is clear that reference compound **2.1** typically leads to a higher inhibition of the target protein activity as compared to the tetrazines of Formula (1), which show a lower inhibition. This advantageous effect of the compounds of Formula (1) is especially apparent when looking at the inhibition of COX-1, ACES, and calcium channel L-type, dihydropyridine.

Table 1. *Results from the enzyme inhibition studies.*

Tetrazine	% COX-1 inhibition	% MAO-B inhibition	% ACES inhibition	% Calcium channel L-type dihydropyridine inhibition

2.1 (reference)	59	36	44	44
2.4	53	20	11	10
2.5	18	9	-1	1
2.7	37	38	22	-17
2.10	14	22	5	-3

Example 4: maximum tolerated dose (MTD) of tetrazines in mice

In this Example, groups of Swiss albino mice (an equal number of male and female animals) were subjected to a single dose of tetrazine **2.1** (reference), **2.4**, **2.5**, **2.7**, or **2.10**. Different groups were used for different doses. For example, for the doses 8, 39, and 78 µmol/kg of reference compound **2.1** three different groups of mice were used. Control groups were also used, wherein mice were only administered vehicle (phosphate-buffered saline (PBS) pH 7.4) without tetrazine ("vehicle control").

Mice were treated in line with ethical guidelines. For example, mice were maintained and monitored for good health at the discretion of a laboratory animal veterinarian, certified rodent diet was provided *ad libitum*, and water was available *ad libitum*. Environmental controls for the animal room were set to maintain a temperature of 22 to 25°C, humidity of 30-70% RH, and a 12-h light/12-h dark cycle.

The dose formulations were prepared as follows. Tetrazine **2.1** (reference), **2.4**, **2.5**, **2.7**, or **2.10** was dissolved in PBS pH 7.4 to prepare separate stock solutions of each tetrazine. The pH of the above solutions was adjusted with 2M sodium carbonate to 7.17. Further dilutions of the tetrazine stock solutions were done with PBS pH 7.4 to achieve a desired concentration.

Mice were dosed on day 1 by intravenously administering the dose formulation (expressed in µmol of tetrazine per kg body weight of the mouse). Thereafter, the mice were observed up to 72 hours post-dose for any signs of mortality. The number of dead mice after 72 hours ("mortality") was then divided by the original number of mice.

The results of these experiments are summarized in Table 2 below. Therein, it is clear that the maximum tolerated dose of reference compound **2.1** is about 39 µmol/kg. By contrast, for the tetrazines of Formula (1) (**2.4**, **2.5**, **2.7**, and **2.10**) the maximum tolerated dose is at least 57 µmol/kg. The improvement as compared to reference compound **2.1** is especially evident for compounds **2.5**, **2.7**, and **2.10** which have MTD's of at least 236 µmol/kg.

Table 2. *Results from maximum tolerated dose studies in mice.*

Tetrazine	Tetrazine dose (µmol/kg)	No. of mortality vs. total no. of mice
No tetrazine (vehicle control)	N/A	0 out of 6
2.1 (reference)	8	0 out of 6
	39 (**MTD**)	0 out of 6
	78	4 out of 6
2.4	19	0 out of 6
	57 (**MTD**)	0 out of 6
	95	4 out of 4
2.5	85	0 out of 6
	171	0 out of 6
	512 (**MTD**)	0 out of 6
2.7	103	0 out of 6
	207	0 out of 6
	621 (**MTD**)	0 out of 6
2.10	118	0 out of 6
	177	0 out of 6
	236 (**MTD**)	0 out of 6

Example 5: general *in vitro* and *in vivo* properties

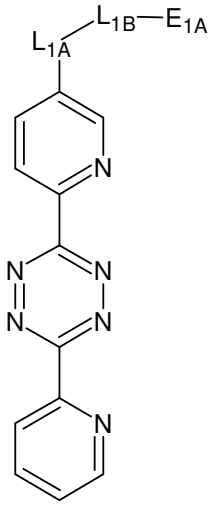
Compounds **2.4**, **2.5**, **2.7**, and **2.10** of Formula (1) were also tested for their:

i. *in vitro* and/or *in vivo* reactivity towards *trans*-cyclooctenes in relation to payload release from said *trans*-cyclooctene;
ii. *in vitro* stability in mouse, rat, and human plasma;
iii. *in vitro* stability in presence of mouse, rat, and human microsomes;
iv. cell toxicity using LS174T colon carcinoma cells;
v. passive membrane permeability at pH 7.4 using a Parallel Artificial Membrane Permeability Assay; and/or
vi. genotoxicity (*i.e.* mutagenecitiy) in *S. typhimurium* strains TA98, TA100, TA1535 and 1537; and in *E. coli* strain WP2 uvrA[pKM101] in presence or absence of S9.

All experiments were carried out using standard protocols known in the art. In all cases, the compounds of Formula (1) showed desired results. In particular, these results were comparable to those obtained for **2.1** when **2.1** was subjected to the same tests i-vi.

Claims

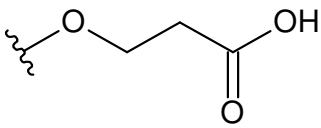
1. A compound or a salt, hydrate, or solvate thereof; wherein said compound has a structure according to Formula (1):



Formula (1); wherein

L_{1A} and L_{1B} are independently selected linkers;

E_{1A} is selected from the group consisting of:



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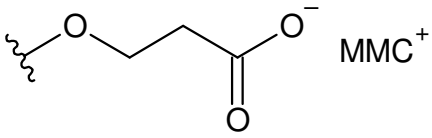

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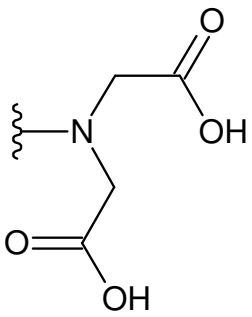

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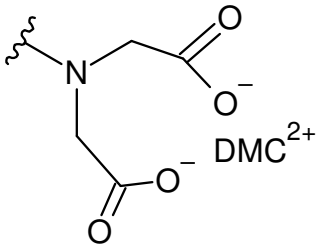
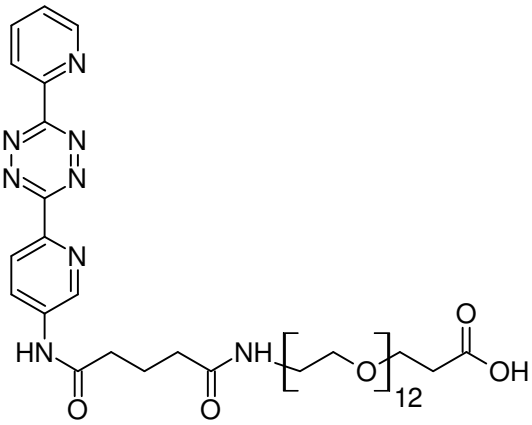


; wherein

MMC^+ is a monovalent metal cation;

DMC^{2+} is a divalent metal cation;

with the proviso that the compound of Formula (1) is not



or a salt, solvate, or hydrate thereof;

preferably MMC^{+} is Na^{+};

preferably DMC^{2+} is Ca^{2+}.

2. The compound according to claim 1, or the salt, hydrate, or solvate thereof; wherein L_{1A} is a linker containing at most 10 atoms.

3. The compound according to any one of the preceding claims, or the salt, hydrate, or solvate thereof; wherein L_{1A} is selected from the group consisting of -O-, -S-, -SS-, -NR_{L1A}-, -N=N-, -C(O)-, -C(O)NR_{L1A}-, -OC(O)-, -C(O)O-, -OC(O)NR_{L1A}-, -NR_{L1A}C(O)-, -NR_{L1A}C(O)O-, -NR_{L1A}C(O)NR_4-, -SC(O)-, -C(O)S-, -SC(O)O-, -OC(O)S-, -SC(O)NR_{L1A}-, and -NR_{L1A}C(O)S-; wherein

R_{L1A} is hydrogen or $C_{1\text{-}3}$ alkyl;

preferably L_{1A} is -NR_{L1A}C(O)-;

preferably R_{L1A} is hydrogen.

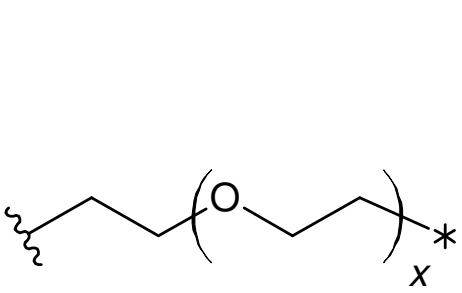
4. The compound according to any one of the preceding claims, or the salt, hydrate, or solvate thereof; wherein L_{1B} is a linker containing at most 50 atoms;

preferably L_{1B} is a linker containing at most 30 atoms;

more preferably L_{1B} is a linker containing at most 25 atoms;

most preferably L_{1B} is a linker containing at most 10 atoms.

5. The compound according to any one of the preceding claims, or the salt, hydrate, or solvate thereof; wherein L_{1B} is selected from the group consisting of -CH_2-,



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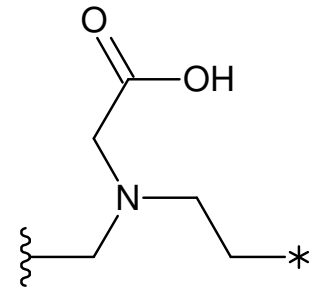

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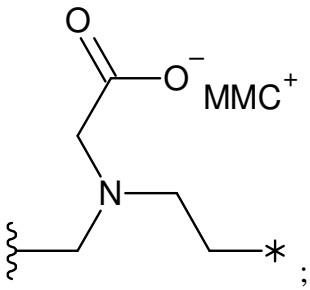


;

wherein

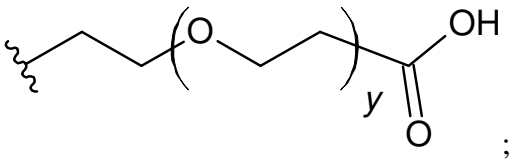
the wiggly line indicates a bond to L_{1A} and the asterisk a bond to E_{1A};

x is an integer in a range of from 1 to 12;

preferably x is an integer in a range of from 2 to 7;

more preferably x is an integer in a range of from 2 to 4.

6. The compound according to any one of the preceding claims, or the salt, hydrate, or solvate thereof; wherein L_{1B} and E_{1A} together form a moiety selected from the group consisting of:



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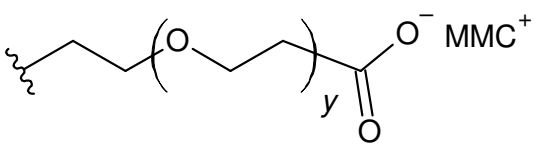

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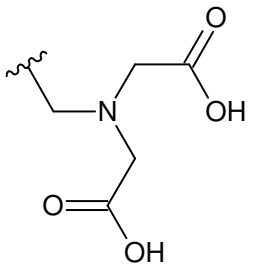

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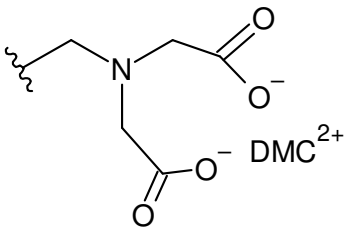

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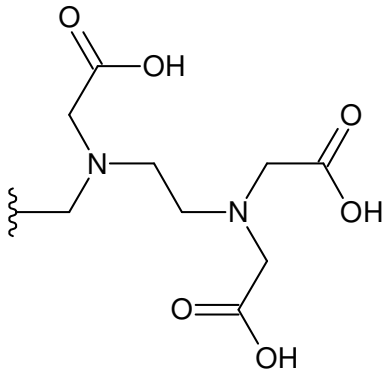

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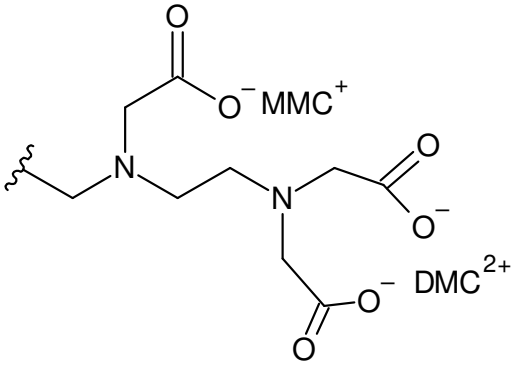


;

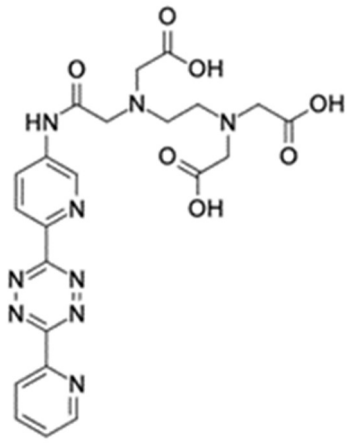
wherein

y is an integer in a range of from 1 to 13;

preferably y is an integer in a range of from 2 to 6;

more preferably y is an integer in a range of from 2 to 4.

7. The compound according to any one of the preceding claims, or the salt, hydrate, or solvate thereof, wherein said compound is selected from the group consisting of:



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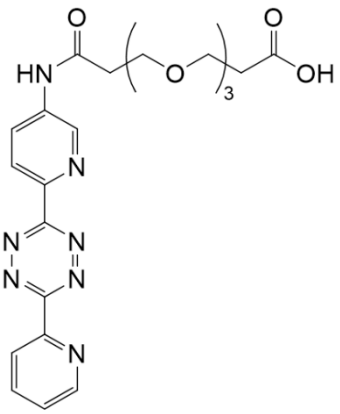

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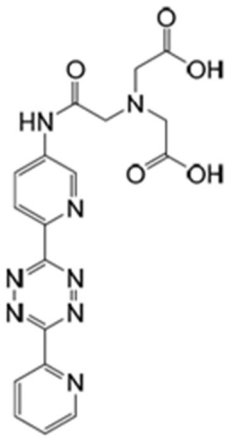

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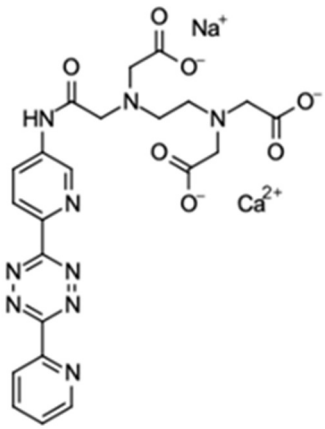
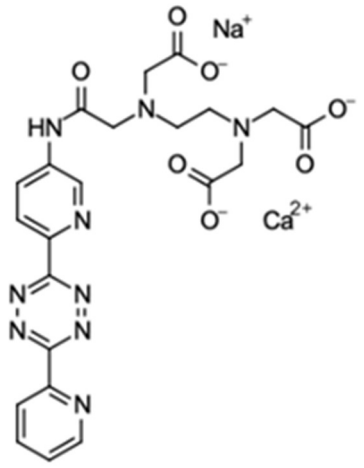


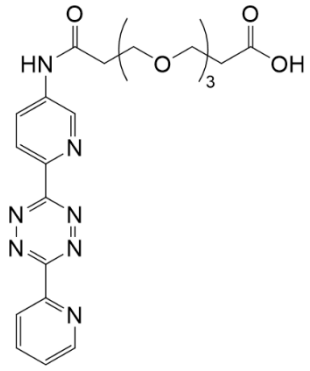
.

8. The compound according to any one of the preceding claims, or the salt, hydrate, or solvate thereof, wherein said compound is:



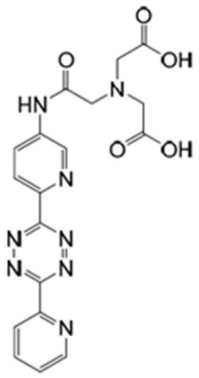
.

9. The compound according to any one of claims 1 to 7, or the salt, hydrate, or solvate thereof, wherein said compound is:



.

10. The compound according to any one of claims 1 to 7, or the salt, hydrate, or solvate thereof, wherein said compound is:



.

11. A composition comprising a compound according to any one of claims 1 to 10, or the salt, hydrate, or solvate thereof;

preferably the composition is a pharmaceutical composition.

12. A combination of

(A1) a compound according to any one of claims 1 to 10, or the salt, hydrate, or solvate thereof; and/or

(A2) a composition according to claim 11; with

(B) a dienophile or a salt, solvate, or hydrate thereof;

preferably the dienophile comprises a *trans*-cyclooctene moiety.

13. The compound according to any one of claims 1 to 10, or the salt, hydrate, or solvate thereof; the composition according to claim 11; or the combination according to claim 12; for use as a medicament.

14. The compound according to any one of claims 1 to 10, or the salt, hydrate, or solvate thereof; the composition according to claim 11; or the combination according to claim 12; for use in the treatment of a disease in a subject,

preferably the subject is a human;

preferably the disease is cancer.

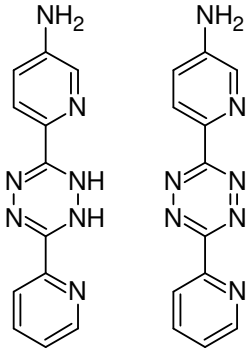
15. A method for preparing a compound according to claim 7, wherein said method comprises the steps of:

(a) reacting SM1a or SM1b with a reagent selected from the group consisting of SM2, SM3, and SM4;

(b) if SM1a is used in step (a), subjecting the reaction product of step (a) to oxidation;

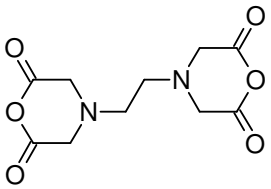
(c) optionally, subjecting the reaction product of step (a) or step (b) to salt formation;

wherein SM1a, SM1b, SM2, SM3, and SM4 are:

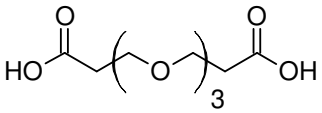


SM1a

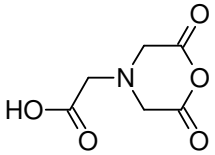
SM1b



SM2



SM3



SM4

;

preferably in step (a) SM1a is used;

preferably in step (b) the reaction product of step (a) is contacted with sodium nitrite.

16. A non-therapeutic method for reacting:

(ia) the compound according to any one of claims 1 to 10, or the salt, hydrate, or solvate thereof; and/or

(iia) the composition according to claim 11;

with a dienophile or a salt, solvate, or hydrate thereof,

wherein said method comprises the step of contacting (ia), and/or (iia) with said dienophile or salt, solvate, or hydrate thereof,

preferably said non-therapeutic method is an *in vitro* method; and

preferably said dienophile comprises a *trans*-cyclooctene moiety.

17. A non-therapeutic use of:

(a) the compound according to any one of claims 1 to 10, or the salt, hydrate, or solvate thereof;

(b) the composition according to claim 11; and/or

(c) the combination according to claim 12;

in a click reaction.

18. A method of treating a disease in a subject, wherein said method comprises the step of administering to said subject:

(a) the compound according to any one of claims 1 to 10, or the salt, hydrate, or solvate thereof;

(b) the composition according to claim 11; and/or

(c) the combination according to claim 12;

preferably the subject is a human;

preferably the disease is cancer.

19. Use of a compound according to any one of claims 1 to 10, or the salt, hydrate, or solvate thereof; the composition according to claim 11; and/or the combination according to claim 12; in the manufacture of a medicament for the treatment of a disease in a subject;

preferably the subject is a human;

preferably the disease is cancer.